# ABC Transporters

**André Goffeau, Benoît De Hertogh and Philippe V. Baret**
*Université Catholique de Louvain, Louvain-la-Neuve, Belgium*

**The ABC proteins constitute the largest family of proteins. They are present in all living species from *Archaea* to *Homo sapiens*. They make up to 4% of the full genome complement of bacteria such as *Escherichia coli* or *Bacillus subtilis*. Each eukaryote genome contains several dozens of members (over 100 in the plant *Arabidopsis thaliana*). They are recognized by a consensus ATP-binding region of approximately 100 amino acids which include the two Walker A and B motifs encompassing a linker or C region (Figure 1). The ABC proteins catalyze a wide variety of physiological functions, most (but not all) of which being related to transport. This article describes the major physiological and biochemical functions as well as the structural properties of some of the best-known ABC transporters using examples from the yeast *Saccharomyces cerevisiae* and *Homo sapiens*.**

## Topology

Most, but not all, ABC proteins are ABC transporters. Each of those molecules contains, or is associated to, one or two cytoplasmic ATP-binding domains named nucleotide binding domains (NBDs) (Figure 1) and one or two transmembrane domains (TMDs) (Figure 2). Each TMD comprises usually six $\alpha$-helix spans. Association of one TMD to one NBD results in a half-size ABC transporter; however, they are believed to function as homo- or heterodimers so that the minimal functional organization of an ABC transporter is considered to be TMD–NBD–TMD–NBD or NBD–TMD–NBD–TMD. In eukaryotes, two TMDs and two NBDs are often associated in one single molecule called full-sized ABC transporter. The topological relation between NBD(s) and TMDs is variable (Figure 2). In bacteria two NBDs often associate with two TMDs either as four single subunits encoded by the same operon or in various combinations of fused subunits. Association of other proteins may occur. The most prominent associated bacterial protein is the periplasmic solute-binding receptor, which in gram-negative bacteria is found in the periplasm, and in gram-positive bacteria is present often as a lipoprotein, bound to the external membrane surface via electrostatic interactions (Figure 3). The three domains of the bacterial ABC uptake transporters: namely the periplasmic binding receptor, the cytoplasmic NDB, and the membrane TMD are believed to have arisen from a common ancestral ABC transporter in which these three proteins were already present. However, during evolution, the sequence of the periplasmic solute-binding receptors diverges more rapidly than that of the TMDs, while that of NBDs is the least divergent. Thus, all NBDs are homologous, but this is not true for the TMDs or the receptors. Nevertheless, the phylogenetic clustering patterns in bacterial ABC from different species are generally the same for all three types of proteins, despite their variable rate of evolution.

The topology of some eukaryotic ABC effluxers can be complex as additional TM spans occur in some systems (Figure 3) as well as extra cytoplasmic domains of presumed regulatory function.

## Phylogeny

The different families of ABC proteins transport a wide variety of substrates against their concentration gradient using the energy of ATP hydrolysis carried out by NBD. In bacteria, the transported substrates are either imported in or exported out of the cell. In eukaryotes, only extracytoplasmic exporters (transporting substrates either out of the cell or into organelles) are known up to now. Within the ABC superfamily, 61 phylogenetic families have been identified so far. These families generally correlate with substrate specificity. Their classification based both on functional and phylogenetic criteria has been carried out within the transporter classification (TC) system developed by Milton Saier in San Diego. The TC system has recently been endorsed by The International Union of the Biochemical and Molecular Biology Societies. In the TC system, prokaryotic ABC influx porters comprise 22 phylogenetic families including histidine permease, the first ABC transporter to be cloned and sequenced in the laboratory of Giovanna Ames in 1982. Another famous example is the MalEFGK operon classified in TC as a maltooligosaccharide porter within "the carbohydrate uptake transporter-1 (CUT1) family," and given the TC digit 3.A.1.1. In this operon, MalE is the receptor, MalF

 © 2004, Elsevier Inc. All Rights Reserved. 

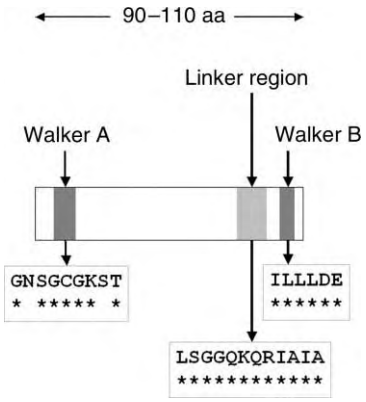



**FIGURE 1** The consensus ATP-binding region of a typical ABC protein is made of approximately 100 amino acids (aa), including both Walker A and B motifs and the linker C region.

and MalG are distinct TMD subunits, and MalK is a double NBD.

The prokaryotic effluxers comprise 27 families including the multidrug exporter LmrA from the gram-positive *Lactococcus lactis* well studied by Will Koning and belonging to "the drug exporter-2 family" (3.A.1.117).

The eukaryotic ABCs can be grouped in only 12 efflux families including the famous MDR1 also named Pgp (permease-glycoprotein), discovered in 1986 by Ira Pastan, Michael Gottesman and colleagues, and shown to be involved in MDR of chemiotreated tumor cells. In the TC system, this ABC exporter is classified in "the multidrug resistance exporter family" (3.A.1.201).

The TC system is redundant with the Human Genome Organization (HUGO) classification adopted by the scientific community working on mammalian objects (mouse or man). The 45 human or mouse ABC proteins, comprising efflux transporters and nontransporter proteins, have been classified in seven families named ABCA to ABCG according to topological and phylogenetical criteria that are less stringent than those used by TC. In its present form, the HUGO nomenclature and classification are difficult to use for identification of novel ABC from non-mammalian species. For instance, the *Saccharomyces cerevisiae* genome contains 32 ABC genes among which 22 (16 full-size and six small-size) are associated to transmembrane domains in four different topologies. Its largest family is the full-sized Pdr5p-like family identified in 1996 by Anabelle Decottignies and André Goffeau and shown later to be present in all fungi and plants. This family is not detected in the animal kingdom. Conversely, the large human and mouse ABCA subfamily is not represented in yeast genomes. There is a necessity to adopt a consistent classification system, which combines the TC and HUGO nomenclatures.

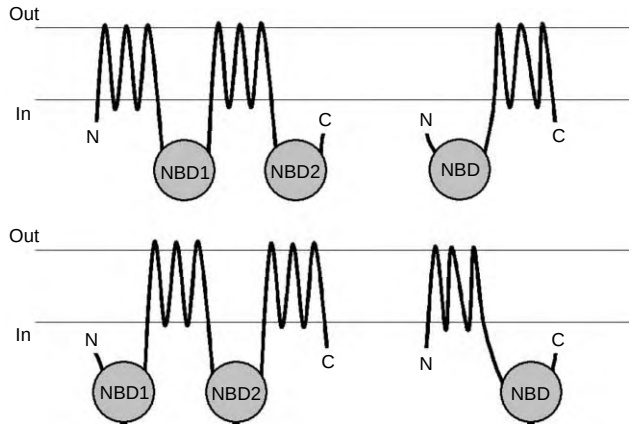



**FIGURE 2** Example of topological relations between NBDs and transmembrane spans in full-sized and half-sized ABC transporters.

## Function and Diseases

The immense variety of substrates transported in bacteria is reflected by the identification of 49 phylogenetic ABC families including 22 influx protein complexes and 27 efflux transporter systems. As they belong to Archaea, gram-negative and gram-positive plasma membranes that are widely different in organization and composition, the number and nature of proteins associated to given ABC transporters are variable and their transport mechanisms may be partly different. In bacterial and Archaea ABC, the variety of substrates: sugars, amino acids, lipids, ions, polysaccharides, peptides, proteins, toxins, drugs, antibiotics, xenobiotics and other metabolites is reflected by the divergence of the periplasmic sensor and that of the TMD, which must control both specificity of substrate and part of the coupling mechanism.

Even if all eukaryotic ABC transporters are effluxers that comprise subunits in which each TMD is fused to a NBD, some of them are not directly involved in moving substrates. For instance, in the cystic fibrosis transmembrane regulator CFTR, and in the sulfonylurea receptor SUR, the hydrolysis of ATP appears to be linked to the regulation of opening and closing of ion channels carried by the ABC protein itself or other proteins (Figure 3B). The conservation of NBD in all ABC transporters, however, suggests that a basic coupling mechanism exists for efflux and influx whatever the transported substrate. Moreover, distantly related proteins exist which utilize an NBD to drive diverse nontransport processes such as DNA repair or protein-elongation or regulation of RNAse activities.

The 32 yeast ABC proteins are in principle easy to study, as sensitive genetic tools are available. However, only a few successful cases of overexpressions and

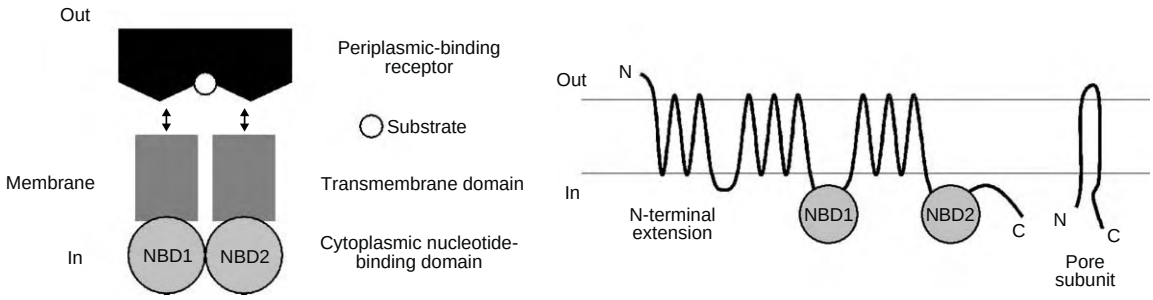



**FIGURE 3** Example of proteins associated to bacterial and mammalian ABC transporters.

*in vitro* UTPase or ATPase measurements of purified ABCs have been reported. Only one purified yeast ABC transporter, the pleiotropic drug resistance effluxer Pdr5p, has been submitted to structural studies.

The biochemical study of human ABC transporters is often more advanced than that of the yeasts. The Pgp protein responsible for multiple drug resistance (MDR) in human cells is especially well studied. One strong impetus for the study of mammalian ABC transporters is their involvement in diseases. Many mendelian diseases and complex genetic disorders are caused by ABC transporters including cystic fibrosis, adrenoleukodystrophy, Stargardt disease, Tangier disease, immune deficiencies, progressive familial intrahepatic cholestasis, Dublin–Johnson syndrome, Pseudoxanthoma elasticum, persistent hyperinsulinemic hypoglycemia of infancy due to focal adenomatous hyperplasia, X-linked sideroblastosis and anemia, age-related macular degeneration, familial hypoapoproteinemia, Fundus flavimaculatis, Retinitis pigmentosum, cone rod dystrophy etc. Cell lines isolated from diseased tissues allow molecular study of the involved ABC transporter. Moreover, a variety of drug-resistant cell lines is available from MDR or MDR-related protein (MRP) tissues. Basic studies of human ABC transporters would greatly benefit from heterologous expression of human ABC transporter genes in yeast or other cells, but this technology is far from being satisfactory yet. Meanwhile, knockout technology in the mouse may be needed to begin to understand the molecular and physiological functions of the mammalian transporters.

## Structure and Biochemical Mechanism

In 1998, the first high-resolution structure of a NBD, that from the histidine ABC importer HisP, was reported. Five years later, about six related structures were available and a consensus view emerged. NBDs are organized as dimers and two molecules of ATP are bound at their interface. Each nucleotide-binding site comprises a Walker A motif from monomer 1 and the C motif from monomer 2. This results from a "head-to-tail" arrangement of the two interacting monomers. This is supported by biochemical arguments and is coherent with the cooperative hydrolysis for ATP hydrolysis observed with MalK.

More recently, three structures of complete dimeric ABC transporters comprising both NDB and TMD were obtained: that of the presumed phospholipid flippase MsbA from *E. coli* and *Vibrio cholera* and that of the vitamin B12 importer BtuCD from *E. coli*. The structures obtained were dissimilar, which may not be too surprising taking into account the different conditions used, the different numbers of TM spans and the different functions (import or export) of the proteins analyzed. No generalization can be made, for instance, on the angle between the TM spans and the membrane plane or on the identification of the interaction domains between the TM spans. The nature of communication between the NBD and TMD is variable and carried out either through the long and complex so-called intracellular domain named ICD in MsbA, or through a short L-shaped linker between the transmembrane spans 6 and 7 in BtuCD. The nature, the size, the orientation, and the location of the so-called chamber (or water channel, or pore, or cone) presumed to be involved in substrate binding are also variable. No consensus interaction points between the NBDs (open or closed conformation) were observed. Obviously, more structures are needed on several transporters carrying out similar functions, such as drug efflux, for instance, to clarify these issues and to reach a consensus interpretation of the basic structural elements involved in the transport and in the coupling mechanism.

In contrast, recent analyses at the electron microscopy level of a bacteria (BmrA or YccV from *Bacillus subtilis*) and a yeast (Pdr5p) drug efflux ABC transporter came to a remarkably coherent set of conclusions. In both cases, the basic structural unit seems to comprise four joining NBDs (that corresponds to two full-size Pdr5p or four half-size BmrA), which are

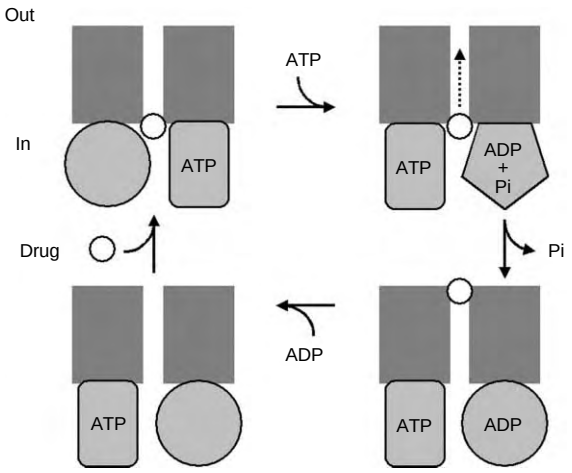



**FIGURE 4** The alternating catalytic sites hypothesis for P-glycoprotein, according to Alan Senior. The NBDs have three ligands and four states: free, ATP bound, ADP + Pi bound, and ADP bound. They alternate in such a way that two ATPs never bind simultaneously. The binding of one ATP to one NBD induces hydrolysis at the other ATP. The drug is transported out during Pi release.

related to the TMDs through four distinct stalks. Each NBD is oriented at a fixed 90° angle relative to its neighbor NBDs. This raises the possibility of concerted rotation movements of the NBDs implying a certain flexibility of the stalks. No intramolecular or no intramembrane pores were observed even though there is room (or chamber) between the four stalks that join together at their NBDs tips.

These latter observations are difficult to reconcile with the mechanism of alternating sites established for the drug exporters, the human Pgp and the bacterial LmrA (Figure 4). In these cases the monomeric NBDs are similar and both are able to hydrolyze ATP. Hydrolysis of ATP at one NBD is believed to be responsible for drug release outside. Upon release of ADP and Pi, the other NBDs bind and then hydrolyze ATP while the drug is taken up inside. However, the simplest version of this "two-cylinder" mechanism cannot function when the two NBDs partners are not equivalent in a dimeric arrangement as it is the cases of yeast Pdr5p and human CFTR, where one of the two NBDs from a full-sized ABC molecule might be unable to hydrolyze ATP.

## The Substrate Specificity of the ABC Multidrug Exporters

One of the most intriguing contemporary biochemical problems is the characterization of the interactions of the TMDs with their transported substrate. The most extraordinary feature in this context is the apparent lack of specificity of the yeast Pdr5p and human Pgp that transports hundreds of different chemicals, apparently contradicting the famous key/slot concept described in all enzymology textbooks. It must be recognized that the identification of presumed ABC substrates are often based on indirect data such as resistance or sensitivity of mutants or drug-induced transcription profiles. These measurements should in principle be corroborated by direct transport measurements, which are often difficult to obtain.

Regarding specificity of transport, one of the best-studied yeast transporters is Pdr5p through the capacity of its deleted mutants to gain growth sensitivity to an amazing variety of xenobiotics. From a large screen of several hundreds of toxic compounds, no chemical determinants for transported substrate specificity could be identified among a wide range of compounds including the fungicides anilinopyrimidines, benzimidazoles, benzenedicarbonitriles, dithiocarbamates, guanidines, imidothiazoles, polyenes, pyrimidynyl carbinols, and strobilurine analogues, the urea derivative and anilide herbicides, a wide collection of flavonoids and steroids, several membrane lipids resembling detergents, and newly synthesized lysosomotropic aminoesters. However, it could be concluded that Pdr5p shows considerable substrate overlap with members of the same ABC phylogenetic family or even with yeast drug effluxers from other families such as Snq2p or Yor1p. This was demonstrated by the numerous cases showing full sensitivity only in double or triple mutants. The most promiscuous substrates were: itraconazole, miconazole, nystatin, antimycin, nigericidin, and tetradodecylammonium bromide, which are transported by at least three distinct yeast ABC transporters. In contrast many substrates show relative specificity for a given pleiotropic drug resistance exporter. Prominent examples include cycloheximide, benomyl, fluxilazole, nuarimal, and soraphen for Pdr5p. From a first large scale screen it was concluded that Pdr5p's most efficient substrates were valinomycin, the antifungal azoles and rhodamine 6G. These compounds are inhibiting the growth of a sensitive PDR5 deletant at concentrations below the micromolar range. Up to now, the most potent competitive inhibitor of binding of rhodamine 6G to the yeast Pdr5p is the oestradiol derivative RU 49953, which exhibits a $K_i$ of 23 nM.

Other recent systematic screens have provided more precise chemical information on the determinants of Pdr5p specificity. From such studies it was concluded that Pdr5p is capable of transporting substrates that neither ionize nor have electron pair donors and that are much simpler in structure than those handled by the human Pgp. The substrate optimum surface volume is about 200Å$^3$. Analysis of the interactions between imidazole derivatives, organotin and other compounds argues, that, as also established for Pgp, the Pdr5p comprises at least two substrate-binding sites. One site

might use only hydrophobic binding interactions. Some substrates may bind to two sites, others associate more specifically to only one site. However, the Pdr5p substrates-binding sites, behave differently from those of Pgp. This concept of overlapping substrate-binding sites may reconcile many previous observations concerning the substrate broad specificity of Pdr5p and Pgp, which up to now appeared contradictory.

## Conclusion

The present frontiers in the study of ABC transporters are challenging. The evolutionary history of this large ubiquitous family has to be unraveled. Additional atomic structures have to be produced. Better heterologous overexpression systems have to be developed to allow further biochemical studies. Specific inhibitors of drug (and other) ABC exporters have to be screened for. The physiological mechanisms of ABC-linked diseases have to be further studied in mouse knockouts. Systems prone to specific inhibition of ABC transporters expression by interfering RNA have to be explored. Genetic therapy has to be developed.

### See Also the Following Article

MDR Membrane Proteins

### Glossary

**human genome organization (HUGO)** An international association that comprises scientists involved in all aspects of the sequence of the human genome and its analysis.

**linker C** Small amino acids sequence signature involved in ATP binding of ABC proteins.

**Pdr5p** The major yeast ABC transporter, involved in pleiotropic drug resistance.

**Pgp** The first ABC transporter (glycoprotein) shown to be involved in mammalian multidrug resistance.

**transmembrane span and transmembrane domain (TMS and TMD)** The existence of the transmembrane span is predicted by frequency of hydrophobic residues in a reading window of about 17 amino acids. In ABC transporters, the transmembrane domains usually comprises six continuous transmembrane spans.

**transporter classification (TC)** Classification of over 800 transporters families, developed by Milton Saier (UCSD), based on a combination of mechanistic and phylogenetic criteria.

**Walker A and B** Small consensus of amino acid sequences involved in ATP binding.

### Further Reading

Dean, M. (2002). *The Human ATP-Binding Cassette (ABC) Transporter Superfamily.* Monograph Bethesda (MD), NCBI, National Library of Medicine (US).

Decottignies, A., and Goffeau, A. (1997). Complete inventory of the yeast ABC proteins. *Nat. Genet.* **15**, 137–145.

Gottesman, M. M., and Ambudkar, S. V. (2001). Overview: ABC transporters and human disease. *J. Bioenerg. Biomembr.* **33**, 438–453.

Hipfner, D. R., Deeley, R. G., and Cole, S. P. C. (1999). Structural mechanistic and clinical aspects of MRP1. *Biochim. Biophys. Acta* **1461**, 359–376.

Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, U., Muller-Rober, B., and Schulz, B. (2002). Plant ABC transporters: more than just detoxifiers. *Planta* **214**, 345–355.

Schmitt, L., and Tampé, R. (2002). Structure and mechanism of ABC transporters. *Curr. Opin. Struct. Biol.* **12**, 754–760.

Senior, A. E., al-Shawi, M. K., and Urbatsch, I. L. (1995). The catalytic cycle of P-glycoprotein. *FEBS Lett.* **27**, 285–289.

### Biography

André Goffeau is Emeritus Professor at the Université Catholique de Louvain (Belgium), Department of Physiological Chemistry. He investigates the P-type ATPases and the ABC transporters in *S. cerevisiae*. He has initiated and organized the sequencing of the yeast genome. He is interested in the phylogenetic classification of yeast membrane proteins.

Benoît De Hertogh is Ingénieur Agronome at the Université Catholique de Louvain (Belgium), Department of Quantitative Genetics. He is completing a Ph.D. thesis on population genetics on the genetical basis of fertility in livestock. He is interested in comparative genomics of membrane proteins.

Philippe V. Baret is Associate Professor at the Université Catholique de Louvain (Belgium), Department of Quantitative Genetics. He is modeling the relationship between genes and phenotypes, with a peculiar interest in quantitative trait loci mapping and cladistic approaches.

# Abscisic Acid (ABA)

**Ramanjulu Sunkar and Jian-Kang Zhu**
*University of Arizona, Tucson, Arizona, USA*

**Plants perceive a variety of environmental and endogenous signals, which elicit appropriate responses with altered metabolism, growth, and development. Phytohormones are signaling molecules, present in trace quantities and are tightly controlled by various biosynthetic, catabolic, and conjugation pathways. Changes in hormone concentration determine a wide range of plant responses, some of which involve interactions with environmental factors. One such important phytohormone is abscisic acid (ABA), a sesquiterpenoid (15-carbon) which is partly produced in chloroplasts and other plastids. A low basal level of ABA is required for normal growth and development of plants. However, a dramatic and rapid increase in ABA levels occurs due to *de novo* synthesis in vegetative parts of the plants exposed to osmotic stress, as well as in developing seeds during maturation. Elevated levels of ABA play a central role in promoting stomatal closure, dehydration tolerance, leaf senescence, seed dormancy and maturation, and coordinated growth of roots and shoots. ABA apparently acts as a signal of reduced water availability. This is manifested at the physiological level, by controlling germination, stomatal movements, and growth. At the molecular level, ABA-dependent changes in gene expression and posttranslational modifications underpin these physiological processes.**

## Mutants As Tools for Studying ABA Biosynthesis and Signaling

Mutant plants with altered biosynthesis, perception, or responses have been crucial in identification of various components involved in ABA biosynthesis and signaling. The genetic screens and selections that have been used include production of nondormant seeds, loss or gain of sensitivity to ABA during germination, seedling or root growth, and altered expression of reporter genes. These approaches have yielded three classes of ABA mutants: ABA-deficient, -hypersensitive, and -insensitive mutants. The characteristic feature of ABA-deficient mutants is a wilty phenotype largely due to impaired stomatal closure (impaired ABA biosynthesis in *aba1/los6*, *vp14*, *aba2*, and *aba3/los5* mutants; *ABA1*, *VP14*, *ABA2*, and ABA3 encode enzymes of ABA biosynthetic pathway, zeaxanthin epoxidase (ZEP), *9-cis* epoxycarotenoid dioxygenase (NCED), short-chain alcohol dehydrogenase/reductase (SDR), and molybdenum cofactor sulfurase (MoCoSu) respectively). Hypersensitive mutants display enhanced sensitivity to ABA, resulting in diminished germination rates at low ABA concentrations and reduced water loss due to enhanced ABA-induced stomatal closure (*Arabidopsis era1, sad1, abh1, hyl1, rop10, and fiery1*). The *ERA1* gene encodes a protein farnesyltransferase; *HYL1*, *SAD1*, and *ABH1* genes encode different types of RNA-binding proteins; *ROP10* encodes a small G protein; and *FIERY1* encodes an inositol polyphosphate 1-phosphatase. In addition to these mutants, silencing of the calcium sensor SCaBP5 and protein kinase PKS3 resulted in ABA hypersensitivity. ABA-insensitive mutants including *abi1*, *abi2*, *abi3*, *abi4*, *abi5*, and *rcn1* from *Arabidopsis*, and *vp1* (orthologue of *abi3*) from maize have been identified. This class of mutants display loss of ABA sensitivity, leading to nondormancy or vivipary. The genes *ABI1* and *ABI2* encode homologous-type 2C protein phosphatases; *RCN1* encodes a 2A-type protein phosphatase; *ABI3/VP1* encodes a B3 type of transcriptional activator; *ABI4* encodes an AP2-type transcription factor; and *ABI5* encodes a b-ZIP transcription factor.

## ABA Biosynthesis

Osmotic stress induction of ABA accumulation is a complex signaling process from the initial water-stress perception, intracellular signal transduction, to gene expression or activation of the enzymes involved in the ABA biosynthesis. The chain of events from the perception of osmotic stress to the gene activation leading to ABA biosynthesis is unknown. Presumably, it involves reactive oxygen species (ROS)/calcium signaling and protein phosphorylation cascades. Significant progress has been made in recent years in cloning the genes encoding enzymes involved in ABA biosynthesis (Figure 1). Some of these genes in *Arabidopsis* are encoded by members of multi-gene families (NCED, abscisic aldehyde oxidase) while others are represented by single gene (ZEP, SDR, MoCoSu). Several of these genes appear to be transcriptionally activated by osmotic stress and ABA.

 © 2004, Elsevier Inc. All Rights Reserved. 

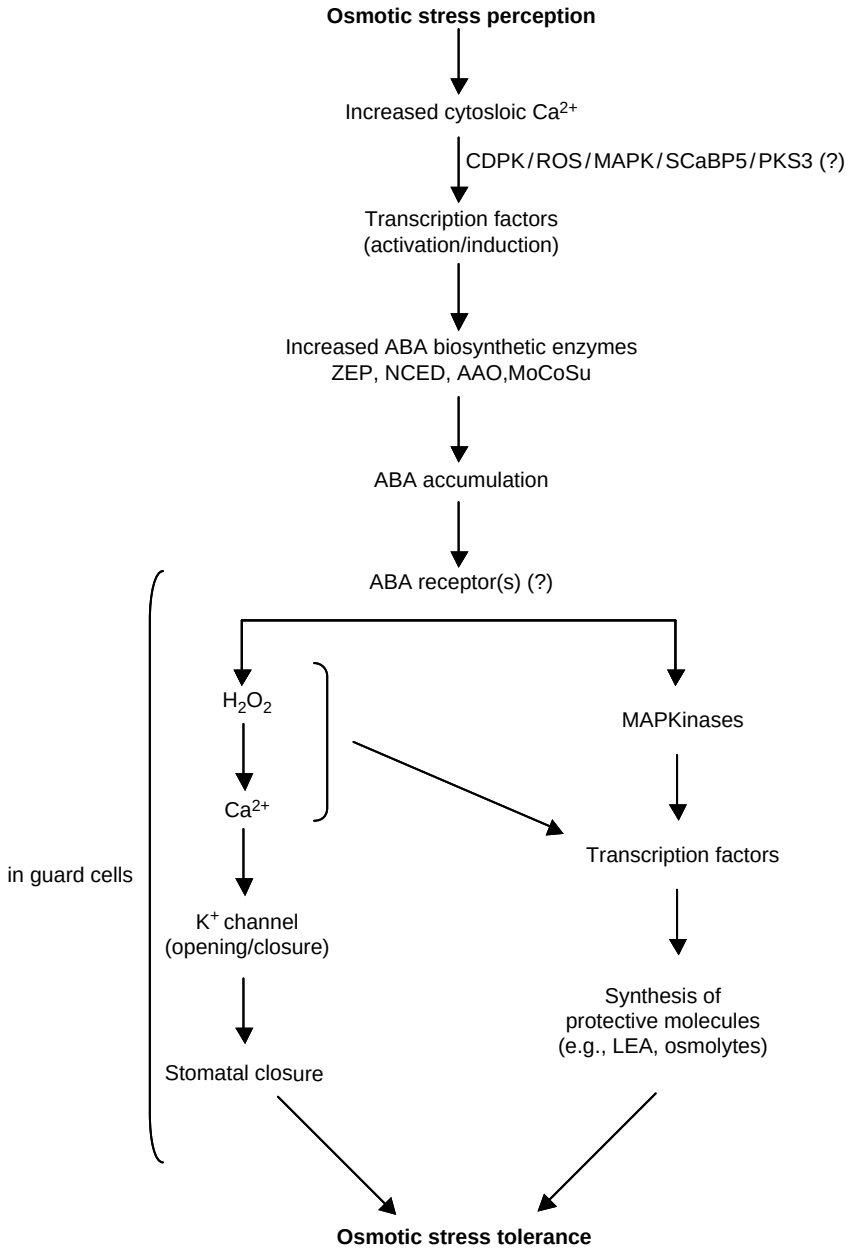



**FIGURE 1** An overview of osmotic stress-induced biosynthesis of ABA and ABA mediated signaling leading to stomatal closure in guard cells and the regulation of gene expression in plants.

ABA biosynthesis in seeds is similar to that in the vegetative tissues. However, the regulation of ABA biosynthesis in developing seeds is not clear since these aspects were mainly investigated in vegetative tissues.

## Role of ABA in Seed Development and Maturation

Seed development represents an important part of the plant's life cycle as the success of seedling establishment to a large extent is determined by the physiological and biochemical properties of the seed. Plant hormones, particularly ABA and gibberellic acid (GA), play antagonistic roles and are important regulators of seed maturation, germination, and postgerminative growth. ABA mediates several important functions in developing seeds such as seed maturation, synthesis of storage proteins, synthesis of late embryogenesis abundant (LEA) proteins, and initiation of seed dormancy, whereas GA is required to break dormancy and to trigger germination. It seems that two peaks of ABA accumulation coordinate these processes in developing seeds. The first peak of ABA is relatively large and occurs

about halfway during seed development (~10 days after pollination) and plays a role in the synthesis of storage proteins. The second peak is relatively weaker compared to the first peak and seems to be important for the synthesis of LEA proteins and for initiation of seed dormancy. During maturation, seeds lose up to 90% of their water content and are still viable. This ability to tolerate extreme dehydration is achieved by ABA-dependent accumulation of LEA proteins and compatible solutes such as sugars. LEA proteins appear to be important for desiccation tolerance by maintaining the structural integrity of membranes and proteins and controlling water exchange. Transcripts encoding either storage proteins or LEA proteins can be precociously induced by ABA in cultured embryos suggesting that endogenous ABA is indeed responsible for these processes. The analysis of promoter sequences for storage proteins and LEA genes corroborated with the ABA responsiveness of *cis*-elements and tissue specificity. Embryo enters a quiescent state (dormancy) at this stage which is also maintained by ABA.

## ABA Signaling in Developing Seeds

ABA signaling components mediating seed dormancy and germination have been inferred from genetic analysis. The best known components involved in ABA signaling in seeds are PP2C-like phosphatases (ABI1 and ABI2) and transcription factors (ABI3, ABI4, and ABI5) and a farnesyltransferase (ERA1). *ABI1* and *ABI2* encode homologous type 2C serine/threonine protein phosphatases. The ABA signaling pathway appears to be negatively regulated by these phosphatases. *ABI3*, *ABI4,* and *ABI5* encode transcription factors of the B3, AP2, and b-ZIP domain families respectively and regulate the expression of ABA-inducible genes in seeds as well as in vegetative tissues. The *ABI3* gene encodes a transcriptional activator with homology to the seed-specific VP1 protein from maize. ABI3 plays a central role in the establishment of desiccation tolerance and dormancy during embryogenesis. ABI3 activates down stream genes and thus is a major player in ABA signaling. VP1 activates expression of ABA-inducible genes through the G-box promoter element. Interestingly, VP1 does not appear to bind to the element directly. Rather, VP1 regulation is likely mediated by protein–protein interactions with G-box-binding factors. In support of this model, the rice TRAB1 b-ZIP protein was shown to interact with rice VP1 in the yeast two-hybrid system. The *Arabidopsis* ABI5 is homologous to TRAB1 and similarly interacts with ABI3. Coexpression of ABI5 and ABI3 transcription factors, which interact physically, synergistically activate several promoters in the presence of ABA. ABI5 plays a role in protecting germinating embryos from drought. ABI5 accumulation, stability, and activity are regulated by ABA during germination. These findings suggest complex interactions of ABI3 and ABI5 in ABA signaling.

## ABA as Mediator of Osmotic Stress Tolerance

ABA has dual roles during osmotic stress: (1) a rapid response in guard cells leading to stomatal closure and (2) a slower response of reprogramming gene expression patterns (Figure 1). Both roles are critical for water-stress tolerance.

### Stomatal Closure Meditated by ABA

Guard cells, which flank stomatal pores, integrate and respond appropriately to changes in water levels mediated by ABA. ABA-deficient and ABA-insensitive mutants are prone to wilting and cannot withstand water-deficit conditions due to their inability to close stomata, while hypersensitive mutants exhibit opposite responses. ABA-induced stomatal closure is vital for plants to limit transpirational water loss during periods of drought. Investigations on ABA-induced stomatal movements have led to the identification of several components acting downstream of ABA. These include protein kinases and phosphatases, phospholipase C and D, slow anion channels, $K^+$ channels, G proteins, sphingosine-1-phosphate, syntaxin, cyclic ADP ribose (cADPR), ROS, and free calcium ions in the guard cell cytosol. However, a complete picture is yet to emerge and there are several missing links.

Stomatal opening and closure are coordinated by regulation of the relative activities of the inward- and outward-rectifying channels and anion channels. ABA-induced stomatal closure is mediated by a reduction in the turgor pressure of guard cells, which requires an efflux of $K^+$ and $Cl^-$, sucrose removal, and the conversion of malate to osmotically inactive starch. Therefore, opening or closing of guard cell ion channels determine the status of stomatal aperture.

#### Second Messengers (ROS and $Ca^{2+}$)

Recent studies have established a role for $H_2O_2$ in ABA signaling through its influence on $Ca^{2+}$ channels in guard cells. ABA stimulates $H_2O_2$ production in guard cells. The plasma membrane NADPH oxidase complex, which consists of many components, seems to be responsible for $H_2O_2$ generation. $H_2O_2$ triggered by ABA plays a vital role as signal mediator for the activation of downstream events including the opening

of $Ca^{2+}$ channels, leading to increases in the cytosolic $Ca^{2+}$ level. It appears that $Ca^{2+}$ plays a very important role in ABA-mediated stomatal closure. ABA-induced changes in cytosolic $Ca^{2+}$ concentration have been attributed to both $Ca^{2+}$ release from internal stores and $Ca^{2+}$ influx from external stores. Downstream to ABA, inositol triphosphate (IP3), cADPR, and possibly inositol hexakisphosphate (IP6) are also implicated in increasing cytosolic $Ca^{2+}$ levels. The highly localized calcium oscillations in the cytoplasm then control various processes leading to ion efflux and stomatal closure. These include inhibition of inward rectifying $K^+$ channels and activation of outward rectifying $K^+$ channels in guard cells, reducing influx and increasing efflux of $K^+$ in guard cells, cation and anion channels in the plasma membrane, changes in the cytoskeleton, and movement of water through water channels in the tonoplast and plasma membrane. The activities of all these cellular structures need to be coordinated during ABA-induced stomatal closure.

ABA responses in guard cells require a specific cytosolic calcium signature. Guard cell cytosolic calcium oscillations with defined frequency and amplitude result in stomatal closure. In contrast, steady and sustained cytosolic calcium increases without oscillations fail to confer stomatal closure, suggesting that excessive, nonoscillating cytosolic calcium may interfere with or inhibit ABA responses. Hence in addition to calcium sensors as positive regulators of downstream ABA signaling, there may be calcium-sensing systems that regulate ABA responses negatively. Consistent with this hypothesis, a calcium sensor protein, SCaBP5, and a protein kinase, PKS3, involved in ABA signaling have been identified recently. *Arabidopsis* loss-of-function mutations in either of these genes resulted in ABA hypersensitivity during seed germination, vegetative growth, and stomatal closure.

### Protein Phosphatases

Phosphorylation events are central to ABA signaling. The *abi1-1* and *abi2-1* mutations confer ABA insensitivity in seed germination, vegetative growth, and the expression of certain ABA-regulated genes. The fact that ABI1 and ABI2 are protein phosphatases suggests involvement of protein phosphorylation/dephosphorylation in regulating ABA signaling. Protein phosphatase genes *ABI1* and *ABI2* encoding type-2C protein phosphatases are up-regulated by ABA. These phosphatases, in general, are implicated in negative regulation of ABA signaling.

### Kinases

Protein kinases that are specifically activated by ABA offer better understanding of the ABA signaling in plant cells. PKABA1 (abscisic acid-responsive protein kinase) from wheat, open stomata 1 (OST1) from *Arabidopsis*, and abscisic acid-activated protein kinase (AAPK) from fava bean are ABA-activated serine-threonine protein kinases. Both AAPK and OST1 display ABA-dependent autophosphorylation. Mutations in the *OST1* gene suppressed ABA-induced ROS production, which is important for stomatal closure, suggesting that OST1 is an important component acting upstream of ROS generation in ABA signaling. Dissection of guard cell signaling events identified a target for AAPK. AAPK activates AKIP1, which is highly similar to a single-stranded RNA-binding protein that binds heterogenous nuclear RNA (HnRNA). HnRNA-binding proteins are a class of proteins involved in transcriptional, posttranscriptional, and translational control of gene expression. AKIP1 is phosphorylated by AAPK and the phosphorylation activates AKIP1 and increases its affinity for a dehydrin (LEA-type) mRNA.

Kinases that act as negative regulators of ABA signaling have been identified. Silencing of PKS3 caused ABA hypersensitivity in seed germination, stomatal closing, and gene expression, suggesting that this protein acts as negative regulator that specifically modulates ABA signal transduction. These findings highlight the existence of ABA-dependent kinases that function both in guard cell signaling and in gene expression.

### Farnesyltransferase

Farnesyltransferases influence protein structure or localization through mechanisms other than phosphorylation. Protein farnesylation, a posttranslational modification process, mediates the COOH-terminal lipidation of specific cellular proteins such as Ras and other G proteins. Deletion of the *Arabidopsis* farnesyltransferase gene *ERA1* or application of farnesyltransferase inhibitors resulted in ABA hypersensitivity of guard cell anion channel activation and of stomatal closing.

### Cytoskeleton

ABA-induced stomatal closure requires a reorganization of the actin cytoskeleton of guard cells. A link for such an action has been demonstrated between ABA and actin through AtRac1. AtRac1 (*Arabidopsis* Rho-related small guanosine triphosphatase, GTPase) has been identified as a central component in ABA-mediated stomatal closure. In animals and yeast, Rho GTPases are key regulators of the actin cytoskeleton. GTPases are inactivated by ABA treatment leading to the disruption of guard cell actin organization. ABA-induced increase in cytosolic $Ca^{2+}$ and the cytoskeleton reorganization seems to be another link in this pathway, suggesting that the ABA-induced increases in cytosolic

$Ca^{2+}$ regulate cytoskeletal reorganization, in addition to ion channels.

### RNA-Associated Proteins and ABA Signaling

Accumulation of some of the ABA-inducible proteins is also dependent on posttranscriptional regulation. RNA processing proteins may contribute to this level of regulation. Isolation of three ABA hypersensitive mutants *abh1*, *hyl1*, and *sad1* all encode RNA-associated proteins implicating that RNA processing modulates ABA signal transduction. The *hyl1* mutant shows ABA hypersensitivity during seed germination. The *HYL1* gene is ABA-regulated and encodes a nuclear dsRNA-binding protein. Mutation in this gene alters the plant responses to several exogenous hormones. The *abh1* mutation confers ABA hypersensitivity in seed germination, stomatal closure, and ABA-induced guard cell calcium increases. ABH1, an mRNA cap-binding protein, is a negative regulator of ABA responses including stomatal closure. The *sad1* mutant is not only hypersensitive to ABA but also to drought, unlike *abh1* and *hyl1* which are drought tolerant. *SAD1* encodes an Sm-like SnRNP protein required for mRNA splicing, export, and degradation. Similarly, AKIP1 is another RNA-binding protein that belongs to this group of proteins involved in ABA signaling. How these proteins execute their role in the ABA pathway is unknown but the findings provide a strong link between RNA processing and ABA signaling.

## ABA-Induced Modulation of Gene Expression

The synthesis of endogenous ABA increases in response to osmotic stress, which in turn induces numerous stress-associated genes involved in the accumulation of osmoprotectants and LEA proteins, protein turnover, stress signaling pathways, and transcriptional regulation. ABA accumulated in response to osmotic stress is likely to be sensed by a receptor protein (sensor), albeit the identity and the location of such a component is yet unknown. The components of ABA signaling involved in transcriptional and posttranscriptional regulation have been less understood as compared to the guard cell signaling. MAPK cascade is an extensively used pathway in eukaryotic signaling. ABA signaling in plants may also use an MAPK pathway. MAPKs (AtMPK3 and AtMPK6) that are activated by ABA are known in plants. These MAPKs receive signals from the ANP1 family of MAPKKKs through MAPKKs (AtMKK4 and AtMKK5). However, a functional link between ABA and MAPK pathway is unknown, although ABA-dependent phosphatidic acid generated by phospholipase D may be responsible for the activation of the MAPK pathway. In addition to the MAPK pathway, CDPKs may also be involved in transmitting the ABA signal to the transcriptional machinery.

### Transcription Factors

Several classes of transcription factors emerged as targets of ABA signaling events and comprise members of the basic leucine zipper proteins (b-ZIP), AP2, Myb, Myc (basic helix-loop-helix, b-HLH), and homeodomain-containing leucine zipper proteins (HD-ZIP). Several of the genes that encode transcription factors or DNA-binding proteins are regulated by ABA, suggesting that a cascade of transcription factors may mediate stimulus-dependent gene expression in ABA responses.

***b-ZIP*** ABFs/AREBs and ABI5 belong to a family of basic leucine zipper class of transcription factors that bind to ABA responsive *cis*-acting elements (ABREs) and confer ABA responsive gene expression. ABF/AREB proteins respond at the transcriptional and posttranscriptional levels to drought and salt stress and appear to be specifically phosphorylated in response to ABA, mediated through ABA-activated protein kinase. ABF3 and ABF4/AREB2 overexpression in transgenic *Arabidopsis* plants resulted in ABA hypersensitivity, enhanced drought tolerance accompanied with decreased transpiration, suggesting that ABF3 and ABF4 are involved in stomatal closure mediated by ABA. Promoters of both *ABF3* and *ABF4* were found to be most active in roots and guard cells, consistent with their roles in stomatal regulation and water-stress response. Some of the target genes for b-ZIPs are LEA, and other ABA-dependent genes.

***MYB and MYC*** Regulation of expression of many ABA-inducible genes has been postulated to involve the ABRE *cis*-elements in their promoter regions. However, the RD22 promoter does not contain any typical ABRE consensus sequence but still is activated by ABA, suggesting the possible involvement of other regulatory elements responding to ABA other than the ABRE-b-ZIP regulatory module. The *Rd22* promoter contains MYC- and MYB-binding elements responsible for drought and ABA activation. The transcription factors that bind these elements, AtMYB2 and AtMYC2 are able to induce *RD22* gene expression in response to drought and ABA. Consistent with their roles, transgenic plants overexpressing AtMYC2 and/or AtMYB2 are hypersensitive to ABA. Hypersensitivity to ABA was enhanced in transgenics when both genes are overexpressed together. On the other hand, knockout mutant in *AtMYC2* showed insensitivity to ABA.

***HD-ZIP*** A number of HD-ZIP proteins have been suggested to be dependent on ABA signaling for their transcriptional regulation. *Arabidopsis* HD-ZIPs, *ATHB5*, *ATHB6*, *ATHB7*, and *ATHB12* are induced

by the exogenous application of ABA or abiotic stresses that cause ABA accumulation, indicating that these transcription factors form part of the ABA signaling. The target genes induced by HD-ZIPs are unknown.

### ABRE (Abscisic Acid Responsive Elements)

Exploration on more downstream elements of ABA signaling in stress responses, especially drought and cold tolerance, identified ABREs in promoters of ABA-induced genes. In most cases, the ABREs contain a core ACGT motif, the most common of those is designated the G-box (CACGTG) that is recognized by b-ZIP transcription factors. The ABRE functions efficiently when two copies are located tandemly or when it is associated with a coupling element. *Cis*-elements called coupling elements which are active in combination with an ABRE but not alone have also been identified. In the promoters of barley genes *HVA22* and *HVA1*, the coupling elements CE1 and CE3 (ACGCGTGTCCTG) are necessary for activation by ABA. Dissection of these promoters defined ABA responsive complexes (ABRCs) consisting of a coupling element and an ABRE capable of conferring ABA inducible transcription.

## See Also the Following Articles

Calcium Signaling: Cell Cycle • Mitogen-Activated Protein Kinase Family • Photosynthetic Carbon Dioxide Fixation • Protein Kinase B • Protein Kinase C Family • Protein Tyrosine Phosphatases • Small GTPases

## Glossary

**abscisic acid** Terpenoid compound, one of the plant hormones.

**phytohormone** Compound that is synthesized by a plant which regulates growth, differentiation, or other specific physiological processes.

**seed dormancy** A resting condition of the nongerminating seed with reduced metabolic rate.

**stomata** Microscopic pores surrounded by two crescent-shaped epidermal guard cells.

**water-stress tolerance** Ability to tolerate water-stress conditions.

## Further Reading

Busk, P. K., and Pages, M. (1998). Regulation of abscisic acid-induced transcription. *Plant Mol. Biol.* **37**, 425–435.

Federoff, N. V. (2002). Cross-talk in abscisic acid signaling. *Science's STKE* — http://www.Stke.org/cgi/content/full/sigtrans;2002/140/re10.

Finkelstein, R. R., and Rock, C. D. (2002). Abscisic acid biosynthesis and signaling. In *The Arabidopsis Book* (C. R. Somerville and E. M. Mayerowitz, eds.) American Society of Plant Biologists, Rockville, MD.

Ingram, J., and Bartels, D. (1996). The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377–403.

Schroeder, J. I., Kwak, J. M., and Allen, G. J. (2001). Guard cell abscisic acid signaling and engineering drought hardiness in plants. *Nature* **410**, 327–330.

Xiong, L., and Zhu, J.-K. (2003). Regulation of abscisic acid biosynthesis. *Plant Physiol.* **133**, 29–36.

Zhu, J.-K. (2002). Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* **53**, 247–273.

## Biography

R. Sunkar is a Postdoctoral fellow at the University of California, Riverside.

Jian-Kang Zhu is the Director of The Institute for Integrative Genome Biology at the University of California, Riverside. His principal research interest is abiotic stress signaling and tolerance in plants. He holds a Ph.D. from Purdue University and received his postdoctoral training at the Rockefeller University.

# Actin Assembly/Disassembly

**Henry N. Higgs**
*Dartmouth Medical School, Hanover, New Hampshire, USA*

**Actin is a monomeric protein that polymerizes into helical filaments. Apart from its role in muscle cells as a scaffold for myosin-based contraction, actin's function often depends on its ability to assemble into filaments from monomers rapidly, and to disassemble equally rapidly. Actin alone can polymerize *in vitro*, but both the kinetics and equilibria of polymerization are controlled in cells by specific actin-binding proteins that serve to modify the assembly/disassembly cycle inherent to actin itself. Some actin-binding proteins of particular importance are sequestering proteins, profilin, capping protein, and ADF/cofilin.**

## Actin Structure

### Actin Monomer Structures

Actin is a 43 kDa (375 amino acid) globular monomer, which binds a nucleotide (ATP or ADP in cells) in a deep cleft between two halves of the protein (Figure 1A). The affinity of actin for nucleotide is greatly increased by divalent cation. Due to its relative cytosolic abundance (100s of μM), $Mg^{2+}$ is the main actin-bound divalent cation in cells, but other cations, especially $Ca^{2+}$, are used for special purposes *in vitro*.

### Actin Isoforms and Model Systems

Mammals contain six highly conserved actin isoforms, with at least 93% amino acid identity (Table I). Actin from non-mammals is similarly conserved, with budding yeast 88% and *Acanthamoeba castellani* 95% identical to human non-muscle β-actin. Bacteria contain several proteins with some properties similar to actin, Mbl, and Mreb.

Much of the biochemical data on actin assembly/disassembly is derived from studies of vertebrate muscle actin. Seminal studies on actin biochemistry were begun by Straub and Feuer during World War II using rabbit muscle actin. The sole actin isoform of budding yeast has also been a model, due to the combination of biochemistry and genetics in this system.

The highly similar sequences of actins from diverse species suggest that results in these model systems will be close approximations for most actins. However, specific biochemical properties can vary between actins, such as affinities for some actin-binding proteins.

Individual cells can possess multiple actin isoforms simultaneously. Mammalian non-muscle cells often contain both β- and γ-actin. The reasons behind this diversity are not well understood, but differential subcellular localization has been observed. Some unicellular organisms possess multiple actin isoforms. For instance, Dictyostelium has ~20 actin genes.

### Actin Filaments

Actin filaments are right-handed double helices, with ~13 monomers per turn (Figure 1B). Each monomer adds ~2.7Å to the filament, so there are ~370 monomers per μm. All monomers are oriented in the same direction along the helix, making the filament polar. Due to the filament's arrow-like appearance when coated with myosin, the two ends are often called "barbed" and "pointed", respectively. The nucleotide-binding cleft faces the pointed end. Some refer to the barbed and pointed ends as "+" and "−," respectively.

## Actin Dynamics

### General Concepts in Actin Assembly/Disassembly

Under polymerizing conditions, actin monomers spontaneously polymerize into filaments, without need of other proteins. Polymerization from monomers can be broken down to two processes: nucleation, whereby actin monomers assemble to form a trimeric nucleus; and elongation, whereby additional monomers add to this nucleus (Figure 2). Nucleation is highly unfavorable, with equilibrium dissociation constants in the mM range. Consequently, a major control point for cellular actin assembly is enhancement of nucleation rate. Elongation occurs rapidly at the barbed end, (diffusion-limited on-rate) and tenfold more slowly at the pointed end. For reasons explained later, at pointed end elongation rarely occurs in cells, and we will only consider barbed end elongation in this chapter.

 © 2004, Elsevier Inc. All Rights Reserved. 

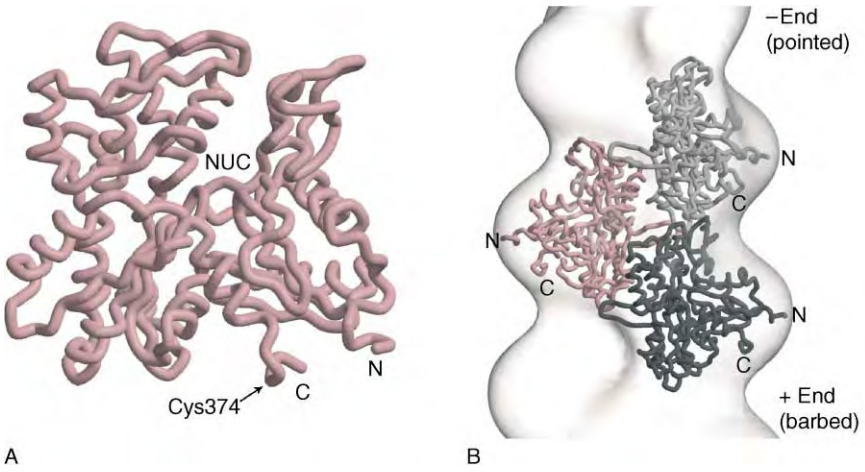



**FIGURE 1** Structure of actin monomers and filaments. (A) Ribbon diagram of actin monomer backbone, oriented with the surface at the pointed end facing upwards. NUC = nucleotide/divalent cation-binding pocket. N and C termini are indicated, as well as cysteine374, which is labeled by pyrene-iodoacetimide. (B) Atomic model of three actin monomers fit into a three-dimensional reconstruction of an actin filament obtained by electron microscopy and image analysis. Both A and B are modified from images provided by Dorit Hanein and Niels Volkmann.

While both Mg-ATP and Mg-ADP monomers can nucleate and elongate, Mg-ATP monomers do both faster. Upon adding to filaments, monomers are referred to as "subunits."

Monomers hydrolyze ATP extremely slowly. Upon adding to a filament, the hydrolysis rate increases to $\sim 0.3\ \text{sec}^{-1}$. With a reasonable concentration of ATP-actin monomers, ATP hydrolysis occurs more slowly than elongation, which occurs at $10\ \mu\text{M}^{-1}\ \text{sec}^{-1}$. Thus, a "cap" of ATP-subunits builds up at the barbed end. Furthermore, the inorganic phosphate (Pi) hydrolysis product remains bound to the subunit for a considerable time before dissociating, while the ADP product remains tightly bound while the subunit remains on the filament.

**TABLE I**

**Actin Isoforms**

| Species | Isoform | % identity[a] | GI # |
|---|---|---|---|
| Human | Non-muscle $\beta$ | 100 | 113270 |
| Human | Non-muscle $\gamma$ | 98 | 113278 |
| Human | Skeletal muscle | 93 | 113287 |
| Human | Cardiac | 94 | 113272 |
| Human | Smooth muscle $\alpha$ | 94 | 113266 |
| Human | Smooth muscle $\gamma$ | 93 | 113279 |
| Rabbit | Skeletal muscle | 93 | 71611 |
| Chicken | Skeletal muscle | 93 | 71613 |
| *Drosophila* | Muscle 88F | 95 | 2113792 |
| Budding yeast | Sole Isoform | 88 | 2262056 |
| *Acanthamoeba* | [b] | 95 | 71630 |

[a] To human non-muscle $\beta$.
[b] Number of actin isoforms unknown.

At steady state, three regions exist on an actin filament: ATP-subunits at the barbed end; ADP-Pi-subunits in the middle; and ADP-subunits toward the pointed end. As discussed later, Pi release is crucial to actin disassembly.

Subunits disassociate from both ends of a filament, and do so more rapidly from the barbed end than from the pointed end. ADP-subunits release from the barbed end faster than ATP-subunits. Once released from the filament, the monomer slowly exchanges its ADP with ATP, assuming an excess of ATP in solution.

Thus, actin assembly/disassembly occurs in a cycle (Figure 2). Nucleation occurs slowly, but filaments elongate rapidly once formed. Since barbed end elongation is tenfold faster than pointed end elongation, and since ATP hydrolysis occurs slowly, ADP-subunits are at higher concentration toward the pointed end. Subunits dissociate from both ends, and do so faster from the barbed end in both the ATP-and ADP-bound states. However, since monomer association is much more rapid at the barbed end, more net growth occurs at this end. Released ADP-monomers exchange ATP for ADP slowly (half-time $\sim 50$ s).

Through this cycle, equilibrium between assembly and disassembly is reached, in which the concentrations of actin in filaments and as monomers become constant. When ATP is in constant supply (and assuming an excess of $Mg^{2+}$ over $Ca^{2+}$), the equilibrium monomer concentration is $\sim 0.1\ \mu\text{M}$ under polymerizing conditions. This monomer concentration, the "critical concentration," is always found in solution at equilibrium under these conditions. The concentration of actin in filaments is the excess above $0.1\ \mu\text{M}$. In other words, given $4\ \mu\text{M}$ actin under polymerizing conditions, $3.9\ \mu\text{M}$ will be in

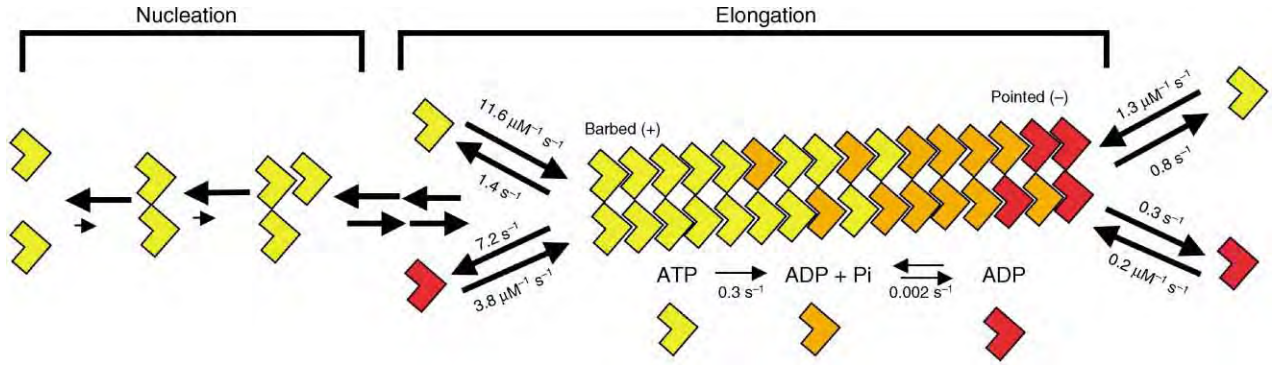



**FIGURE 2** Assembly/disassembly of actin alone. Nucleation is highly unfavorable, signified by the large back arrows in the dimerization and trimerization steps. Once a tetramer is formed, the filament is more stable and monomers add to and disassemble from each end with the indicated kinetic constants (Pollard (1986) *J. Cell Biol*, **103**, 2747–2754). Subunits hydrolyze bound ATP slowly upon addition to filaments, and release the Pi product even more slowly, creating sectors of the filament enriched in ATP-actin (yellow), ADP-Pi-actin (orange), and ADP-actin (red). Upon release from the filament, the ADP on the monomer exchanges slowly with ATP.

filaments and 0.1 μM as monomers. With 100 μM actin, 99.9 μM is in filaments, 0.1 μM as monomers. At or below 0.1 μM, there are no filaments. Even at equilibrium, monomers constantly add to and release from both ends.

### Polymerization Conditions

Three buffer conditions strongly affect actin's propensity to polymerize: ionic strength, the divalent cation present, and pH. Increased ionic strength increases elongation rate. The reason for this effect is not well understood, but thought to be due to masking of charge repulsion between the highly anionic actin monomers. The ionic effect reaches a maximum at around the equivalent of 50 mM KCl, and higher concentrations slow polymerization, probably due to inhibition of specific inter-monomer charge interactions in the filament. As for divalent cation, Mg-actin is better than Ca-actin at polymerizing, due to differences in monomer conformation. For pH, higher pH inhibits polymerization, likely due to increasing the net negative charge of actin, hence increasing charge repulsion between monomers.

## Effects of Actin-Binding Proteins

Actin-binding proteins are widely expressed in eukaryotes, generally at concentrations that exert a significant effect on cellular actin assembly/disassembly. Each protein alters one or more aspects of actin's assembly/disassembly cycle. The basic biochemical functions of these proteins are discussed in this section and illustrated in Figure 3, while their coordinated cellular effects are described in the next section.

### Sequestering Proteins

Sequestering proteins, present in most eukaryotic cells, bind actin monomers and prevent their addition to either end of filaments (Figure 3). Metazoan cells often contain the small protein, thymosin (5 kDa), the most well-characterized isoform being thymosin $\beta$4. Yeast do not have thymosins, but do express twinfilin, which can sequester. Twinifilin is present in mammals and other organisms as well.

### Profilin

Profilin is a 13 kDa protein that binds actin monomers, preferring ATP-actin over ADP-actin about fivefold. Profilin binding has three consequences for actin (Figure 3). First, profilin strongly inhibits spontaneous nucleation. Second, profilin prevents monomer addition to pointed ends. Third, profilin serves as a nucleotide exchange factor for actin, increasing exchange of ATP or ADP at least fivefold, though by some accounts its effect is much greater. A common misconception is that profilin is a sequestering protein. Profilin-bound actin can add to barbed ends at a rate comparable to that of free monomer. An additional feature of profilin is that it binds stretches of polyproline found in many cytoskeletal proteins (e.g., VASP, formins, WASp/Scar/WAVE family proteins, vinculin), binding such proteins and an actin monomer simultaneously.

### Capping Proteins

Capping protein is a heterodimer of 30 kDa subunits that binds filament barbed ends, preventing monomer addition (Figure 3). With barbed ends capped,

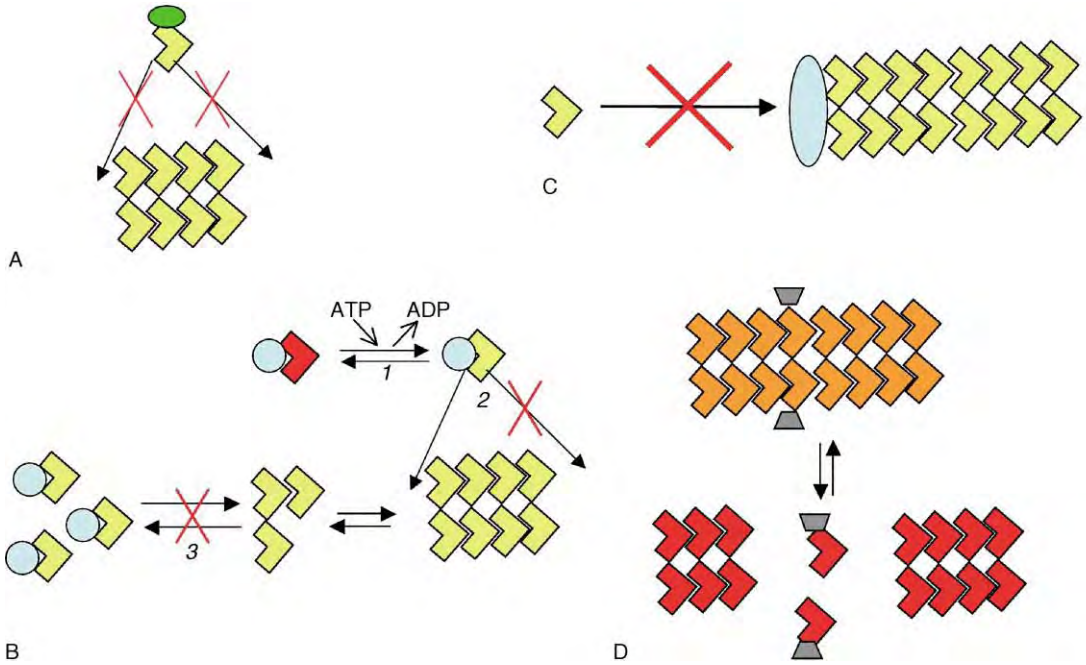



**FIGURE 3** Effects of actin-binding proteins on actin dynamics. (A) Sequestering proteins (green oval) bind actin monomers and prevent addition to either end of the filament. (B) Profilin (light blue circle) binds monomers and has three effects: (1) acceleration of nucleotide exchange on monomers; (2) prevention of monomer addition to pointed ends; and (3) inhibition of spontaneous nucleation. (C) capping proteins (light blue oval) bind filament barbed ends and prevent monomer addition. (D) ADF/cofilin (gray trapezoid) binds ADP-subunits on filaments and promotes Pi release from other subunits. ADF/cofilin also severs filaments. These two activities result in accelerated filament depolymerization.

the critical concentration of actin shifts to 0.7 μM, the critical concentration of the pointed end. Capping protein's high affinity ($K_d < 1$ nM) and slow off-rate (half-life of capped barbed end is 30 min) cause a stable block to barbed end elongation. Members of the gelsolin family also cap barbed ends, in addition to severing filaments.

### ADF/Cofilin

ADF/cofilin is a 15 kDa protein that binds both filaments and monomers, with a 40-fold preference for the ADP-bound state over ATP- or ADP-Pi states in both cases. Mammals contain two isoforms, ADF and cofilin. ADF/cofilin binding to filaments is cooperative, and accelerates Pi release from other subunits on the filament at least 20-fold. ADF/cofilin also severs filaments. ADF/cofilin is often referred to as a depolymerization factor, because its binding to filaments accelerates depolymerization. The mechanism of depolymerization acceleration probably involves both acceleration of Pi release (increasing subunit off-rate from filaments) and severing (creating more ends for depolymerization). However, ADF/cofilin does not prevent polymerization, as ADF/cofilin-bound monomers can still add to filaments.

## Cellular Aspects of Actin Assembly/Disassembly

In mammalian cells, actin concentration is in the 50–200 μM range. Since the critical concentration is 0.1 μM, one would expect the vast majority of actin to be in filaments. However, the ratio of polymerized:unpolymerized actin is often around 1:1 in unactivated cells, while activated cells can reach close to 100% polymerized actin. The 1:1 ratio is maintained largely by the proteins discussed. Many mammalian cells contain more thymosin than actin, as well as a high concentration of profilin (e.g., Neutrophils have ~200 μM actin, 400 μM thymosin, and 100 μM profilin). Despite its lower concentration, profilin binds ATP-actin more tightly than thymosin ($K_d$ of 0.2 μM vs >10 μM), so that monomers distribute between the two proteins. The rapid off-rates of monomers from these proteins (at or above five per second) allow rapid equilibration. Almost all monomers are bound to one protein or the other, leaving ~0.5 μM free actin monomer in cells. This low free monomer concentration virtually eliminates spontaneous nucleation in cytoplasm. Another consequence of these two proteins is that pointed end elongation is effectively suppressed, since thymosin prevents elongation from both ends and profilin prevents pointed

end elongation. For this reason, cellular filaments only elongate from barbed ends (except under specific conditions in muscle cells).

Cellular actin filaments are created by specific nucleation factors. Examples of such factors are Arp2/3 complex and formins. Both nucleation factors are tightly regulated. Thus, cells control the when and where of filament formation.

Mammalian cells contain ~1 μM capping protein, and its high affinity for barbed ends means that new filaments are capped rapidly (~1 s after formation). Capping protein is inhibited by bound polyphosphoinositides, which can promote cellular filament elongation. Some proteins can also inhibit capping protein by competing for barbed end binding, specifically VASP and formins. By controlling capping, cells control filament elongation.

The mechanisms by which cellular filaments disassemble are not understood fully, since off-rates from pointed ends can not explain the rapidity of disassembly in many circumstances. Filament networks 100s of nm thick can disassemble in seconds. ADF/cofilin clearly has a central role in this process, probably both by catalyzing Pi release from filaments and by severing filaments to create new ends for depolymerization. There is, however, evidence that ADF/cofilin may contribute to filament assembly upon initiation of cell motility, perhaps by severing to create new ends capable of elongating and supporting Arp2/3 complex-based nucleation.

The coordinated action of these proteins results in a cycle of assembly/disassembly in cells (Figure 4). Monomers shift from thymosin to profilin, then add to the barbed end. The filament-bound monomer hydrolyzes its ATP and retains ADP and Pi. ADF/cofilin binding to the filament accelerates Pi release and causes filament severing. Monomers dissociate from the pointed end and remain bound to ADF/cofilin. Monomeric ADP-actin exchanges between ADF/cofilin, thymosin, and profilin. Even though ADF/cofilin binds more tightly than profilin to ADP-actin, profilin is at higher concentration (100 versus ~20 μM), and the rapid off-rate from ADF/cofilin enables equilibration. Profilin catalyzes recharging of ATP onto its bound monomer, enabling re-addition of that monomer to the barbed end. Capping protein terminates barbed end elongation except under specific circumstances.

Profilin's ability to bind proline-rich sequences in VASP and formins may have interesting consequences. Both proteins are postulated to reside close to barbed ends of particular filaments and to compete for barbed end binding with capping protein. Binding of profilin-actin at these areas may enable preferential elongation of these filaments even in the presence of capping protein.

The above description is probably accurate for most eukaryotes, including mammals, other vertebrates and multicellular animals, and protozoa. Fungi such as *Saccharomyces cerevisiae* (budding yeast) are somewhat different, containing lower actin and profilin concentrations (5–10 μM for each) and no thymosin. A yeast sequestering protein, twinfilin, is present at unknown concentration. Unlike in vertebrates, most yeast actin (>90%) is polymerized. Yeast contain ADF/cofilin, capping protein, Arp2/3 complex and formins.

Actin assembly/disassembly in plants is poorly understood. Plants contain all the discussed proteins except thymosin. Plant profilin does not catalyze nucleotide exchange.

Other proteins, less well characterized at present, may play significant roles in actin assembly/disassembly.

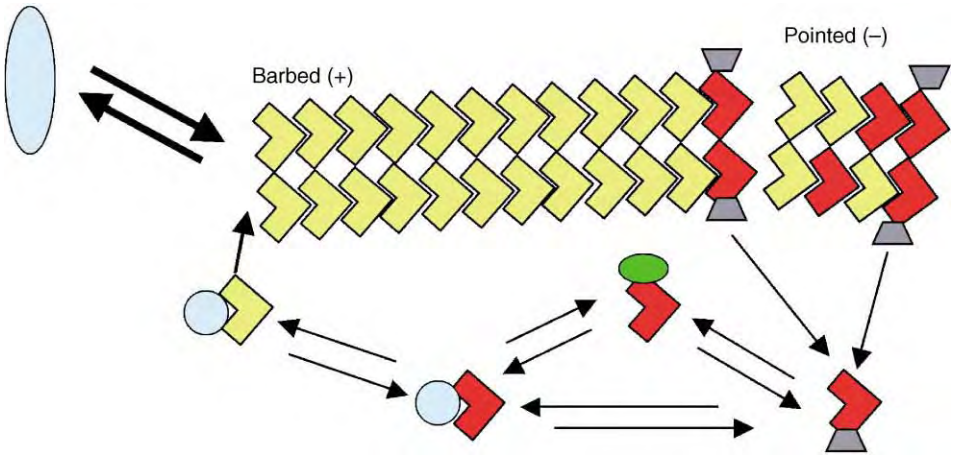



**FIGURE 4** Concerted effects of actin-binding proteins in the assembly/disassembly cycle. Profilin-bound ATP-actin adds to the filament barbed end, then hydrolyzes its ATP slowly. ADF/cofilin binding accelerates Pi release along the filament, and severs the filament. ADF/cofilin-bound ADP-subunits release from filament ends. Profilin, thymosin, and ADF/cofilin compete for binding ADP-monomers. Profilin-bound ADP-monomers exchange ADP for ATP, enhancing their ability to assemble onto barbed ends.

**TABLE II**

**Small Molecule Reagents Used in Actin Research**

| Reagent | Biochemical effect | Cellular effect | Cell-permeant? |
|---|---|---|---|
| Latrunculin A | Sequesters monomer | Depolymerization | Yes |
| Cytochalasins | Cap barbed ends | Depolymerization | Yes |
| Swinholide A | Sequesters dimers, severs filaments | Depolymerization | Yes |
| Phalloidin | Binds filaments | Prevent depolymerization | No |
| Jasplakinolide | Binds filaments | Prevent depolymerization | Yes |

Srv2/CAP complex may accelerate ADF/cofilin-mediated filament depolymerization and monomer recycling. Aip1 may associate with ADF/cofilin and cap barbed ends.

## Reagents Used in Actin Research

### Small Molecules Affecting Actin Dynamics

Table II presents a catalogue of the reagents commonly used in actin research. While this table is simplistic, the effects of some reagents are not, and care must be taken to avoid artifacts. An example is the cytochalasins, a group of compounds commonly used to depolymerize cellular actin. This effect is attributed to the barbed end capping activity of cytochalasins. With barbed ends capped, filaments depolymerize from pointed ends since profilin and thymosin prohibit pointed end elongation. However, at higher concentrations, cytochalasins can have other effects, such as causing ATP hydrolysis on actin monomers through non-productive dimer formation. Use of concentrations below 1 μM largely suppress this secondary effect. In addition, cytochalasin B binds and inhibits a plasma membrane glucose transporter. Despite this property, cytochalasin B is sometimes used in preference to cytochalasin D, because the tenfold lower affinity of cytochalasin B for barbed ends enables more rapid wash-out during cellular recovery experiments.

Latrunculins (isomers A and B) can cause a similar cellular effect to cytochalasins (depolymerizing filaments), but by a different mechanism (sequestering monomers). In neither case do these compounds actively depolymerize filaments. What they do is block polymerization, leaving only depolymerization to occur by the normal cellular mechanisms.

Swinholide A binds/severs filaments, and binds/sequesters dimers, again causing cellular actin depolymerization. This compound has not been widely used in actin cell biology.

Two compounds stabilize filaments: phalloidin and jasplakinolide. Phalloidin is much better characterized. In the presence of phalloidin, the off-rate from barbed and pointed ends drops to near zero, and the critical concentration essentially becomes zero. Phalloidin also slows Pi release significantly. Phalloidin can be labeled with many compounds, including fluorophores, enabling localization of actin filaments in permeabilized cells. TRITC-phalloidin is especially useful, since its fluorescence increases 20-fold upon binding filaments. Since phalloidin is not cell-permeable, jasplakinolide is used for live cell studies. For either compound, concentrations equal to those of actin are necessary to block depolymerization fully, because they bind stoichiometrically along the filament.

## See Also the Following Articles

Actin-Capping and -Severing Proteins • Actin-Related Proteins • Cell Migration • Focal Adhesions • Phosphatidylinositol Bisphosphate and Trisphosphate

## Glossary

**capping protein** In theory, any protein that binds either barbed or pointed end of actin filament and prevents monomer addition to that end. In practice, used to mean barbed end capping protein, and often heterodimeric capping protein in particular.

**critical concentration** The concentration of monomers in a solution of actin at equilibrium between monomers and filaments. Under polymerizing conditions, the critical concentration with both the barbed and pointed ends free is ~0.1 μM. When the barbed end is capped, the critical concentration rises to 0.5–0.7 μM.

**sequestering protein** Protein that binds actin monomer and prevents addition to either end of the filament. Neither profilin nor cofilin are sequestering proteins.

## Further Reading

Amos, L. A., van den Ent, F., and Lowe, J. (2004). Structural/functional homology between the bacterial and eukaryotic cytoskeletons. *Curr. Op. Cell Biol.* **16**, 24–31.

Carlier, M. F., and Pantaloni, D. (1997). Control of actin dynamics in cell motility. *J. Mol. Biol.* **269**, 459–467.

Condeelis, J. (2001). How is actin polymerization nucleated *in vivo*? *Trends Cell Biol.* **11**, 288–293.

Cooper, J. A., and Schafer, D. A. (2000). Control of actin assembly and disassembly at filament ends. *Curr. Opin. Cell Biol.* **12**, 97–103.

Goddette, D. W., and Frieden, C. (1986). Actin polymerization. The mechanism of action of cytochalasin D. *J. Biol. Chem.* **261**, 15974–15980.

Kreis, T., and Vale, R. (eds.) (1999). *Guidebook to Cytoskeletal and Motor Proteins*, 2nd edition. Oxford University Press, New York.

MacLean-Fletcher, S., and Pollard, T. D. (1980). Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. *Biochem. Biophys. Res. Commun.* **96**, 18–27.

Pollard, T. D. (1984). Purification of ADP-actin. *J. Cell Biol.* **99**, 769–777.

Pollard, T. D., Blanchoin, L., and Mullins, R. D. (2000). Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Ann. Rev. Biophys. Biomol. Struct.* **29**, 545–576.

Spudich, J. A., and Watt, S. (1971). The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* **246**, 4866–4871.

Straub, F. B., and Feuer, G. (1950). Adenosine triphosphoate. The functional group of actin. *Biochim. Biophys. Acta.* **4**, 455–470.

## Biography

Henry Higgs is an Assistant Professor of Biochemistry at Dartmouth Medical School. His principal research interest is to determine how cells produce so many different surface protrusions. Since most of these structures depend on actin filaments, he utilizes a great deal of time in research about actin assembly. He holds a Ph.D. in Biochemistry from the University of Washington, and did postdoctoral research at the Salk Institute. Dr. Higgs is a Pew Biomedical Science Scholar.

# Actin-Capping and -Severing Proteins

**Sankar Maiti and James R. Bamburg**
*Colorado State University, Fort Collins, Colorado, USA*

**Actin is one of the major components of the cytoskeleton, a network of protein filaments that organizes the cytoplasm of eukaryotic cells. Actin filaments (F-actin), in the form of a right-handed double-stranded helix, are formed by assembly of globular (G-actin) monomer subunits in a head to tail orientation that gives the filaments a molecular polarity. Based on the orientation of an arrowhead pattern on F-actin decorated with a fragment of the muscle motor protein myosin, one end of the filament is called the barbed end and the other the pointed end. Spatial and temporal regulation of actin-filament assembly, disassembly, and stability in cells drives most aspects of cell motility, cell shape, and the interaction of a cell with its neighbors or surrounding matrix. Actin-capping and -severing proteins are special classes of actin-binding proteins (ABP) that regulate the assembly dynamics of actin filaments. Actin-capping proteins are classified into two groups, the barbed-end-capping proteins and the pointed-end-capping proteins. Depending on their binding affinity and off-rate constants, capping proteins slow down or stop the growth and/or depolymerization of filaments at the end to which they bind. Their interaction with actin may promote nucleation of new filaments, increase branching of filaments, and/or enhance filament depolymerization. Actin-severing proteins bind to the side of actin filaments and enhance fragmentation. Some severing proteins also become barbed-end-capping proteins (e.g., gelsolin) whereas others (e.g., ADF/cofilin) can provide both free barbed and pointed ends that can influence actin dynamics.**

## Actin

Actin is a major cytoskeletal protein in virtually all eukaryotic cells, usually comprising $>1\%$ of cellular protein. In muscle cells where it was first identified, F-actin is the major component of the thin filaments of the contractile apparatus where the myosin motor head groups of the thick filament walk along the actin thin filaments in an energy-dependent process, causing contraction. Mammals have six actin genes, each expressing a different isoform, but the structure of the 42.5 kDa actin monomer is highly conserved across phyla. G-actin has four quasi-subdomains at the center of which is bound an essential adenine nucleotide, either adenosine triphosphate (ATP) or adenosine diphosphate (ADP). As is the case with most molecular self-assembly systems, actin assembly is driven by a positive entropy change that arises from the displacement of water as the subunits come together. Following assembly, bound ATP is hydrolyzed to ADP through an ADP + Pi intermediate. Nucleotide hydrolysis provides the energy input required for actin filaments to maintain equilibrium binding constants for monomers that differ at the barbed and pointed ends. The concentration of monomer required to maintain the filament end is called the critical concentration, and is lower for the barbed end than for the pointed end. Thus, as monomer assembles into filaments and the monomer concentration decreases, the critical concentration of the pointed end is reached first. Monomers still add to the barbed end, leading to monomer loss from the pointed end. This phenomenon is called treadmilling. At steady state there is consumption of energy through ATP hydrolysis that drives the treadmilling of the actin subunits (Figure 1). In some cells this may account for up to 50% of the total ATP consumption. Tropomyosin, a short helical filamentous ABP, binds along the side of actin filaments and covers 5–7 actin subunits. Binding of tropomyosin usually aids in the formation of stable actin filaments, in part because they can inhibit the severing and depolymerizing activity of other ABPs. Dimers of actin are not stable and tend to dissociate but with addition of another monomer, the trimeric actin acts as a nucleus for filament formation. In the cell, spontaneous nucleation of filaments is kept at a minimum by the presence of actin monomer sequestering proteins. Thus, nucleation is a highly regulated event and may be initiated in the cell by three mechanisms: (1) the activation of certain actin binding proteins that help form trimeric nuclei; (2) the removal of capping proteins from the filament barbed ends; and (3) the severing of existing filaments to provide new barbed ends for filament elongation. The sites of nucleation and directionality of actin-filament growth is important for the development of cell polarity and cell migration and is regulated, in part, by the localized activity of capping and severing proteins.

 © 2004, Elsevier Inc. All Rights Reserved. 

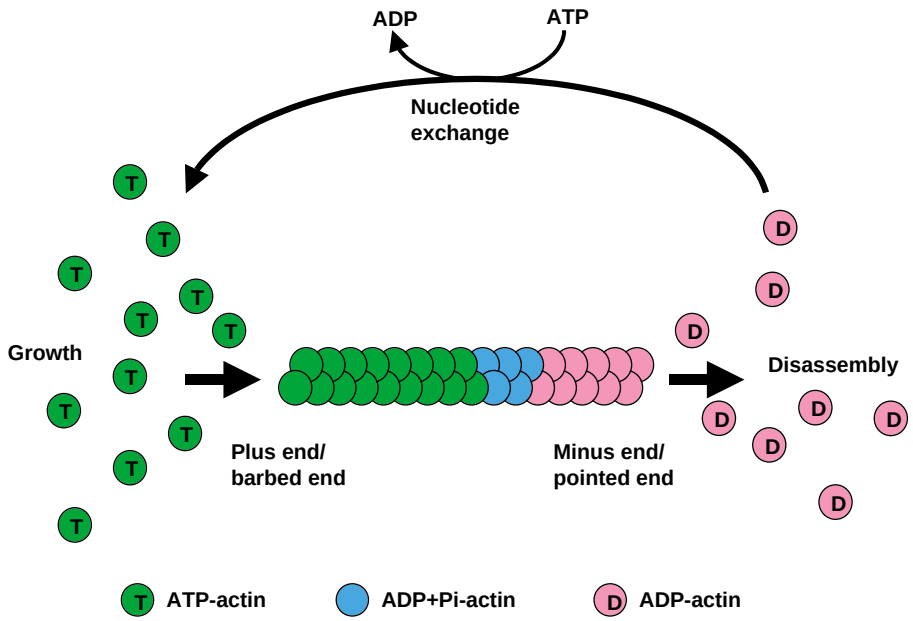



**FIGURE 1** Dynamics of actin filaments at steady-state demonstrating treadmilling. ATP-actin adds onto the barbed end, nucleotide hydrolysis occurs and the inorganic phosphate dissociates leaving ADP-actin subunits to dissociate from the pointed end. Nucleotide exchange of ATP for ADP occurs on the monomer, giving rise to more ATP-actin for filament growth.

## Actin Dynamics at the Leading Edge

Various cell membrane protrusions and inversions, especially lamellipodia, filopodia (microspikes), phagocytic cups, and upward ruffles are distinct morphologies of cells and are important for cell function, motility, and organismal development. These leading edge phenomena are based on actin-filament reorganization. In extending lamellipodia and filopodia, polarized arrays of actin filaments turn over very rapidly, with filament barbed ends facing toward the plasma membrane. The pointed ends of the filaments rapidly depolymerize at the rear of these arrays. In extending lamellipodia, the actin-filament growth is regulated mainly by Ena/VASP, capping protein, and Arp2/3 complex. The Arp2/3 complex causes branching of growing actin filaments and the barbed-end-capping protein limits the growth of newly assembled filaments to maintain short rigid filaments that can serve to push the membrane forward in the broad lamellipodium. Ena/VASP proteins bind to the barbed ends of growing actin filaments and inhibit the binding of Arp2/3 complex and capping protein, leading to the formation of longer and unbranched actin filaments, such as those found in the finger-like filopodial extensions (microspikes). This antagonism in barbed-end binding between Ena/VASP and Arp2/3 complex and capping protein, can help explain the often rapid conversion between lamellipodial and filopodial type of membrane protrusions seen at the leading edge of many migrating cells, especially within the growing tip of neurons, called the growth cone (Figure 2).

## Pointed-End Binding and Branching

Arp2 and Arp3 are two actin-related proteins that form part of a seven-subunit complex known as the Arp2/3 complex, ubiquitous in eukaryotes. The Arp2/3 complex is required for generating the 70° branched actin filaments observed at the leading edge of expanding cell membranes in lamellipodia. There are several possible mechanisms for generating this type of filament array. The Arp2/3 complex may capture the pointed ends of actin filaments created by severing pre-existing

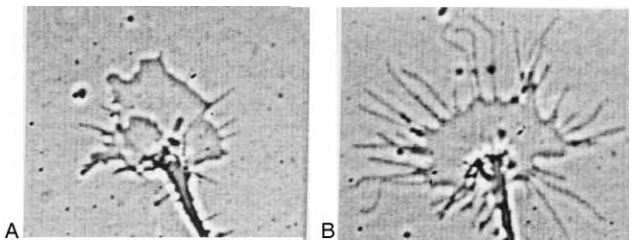



**FIGURE 2** A typical dorsal root ganglion neuronal growth cone. Phase contrast picture in A shows growth cone exhibiting lamellipodial growth. Within a few minutes after reducing the osmolarity of the growth medium, the growth cone exhibits many long and active filopodia (B). Panel width = 44 μm.

filaments and then associate with the side of another pre-existing filament to create a branch point. Alternatively, Arp2/3 complex may bind at the barbed end of a growing filament to create a branch point. The Arp2/3 complex requires activation to become a potent nucleator of actin-filament growth. Activation can occur through the binding of members of the Wiskott–Aldrich syndrome protein (WASP) family, some of which are activated by signaling through G protein-coupled receptors. Arp2/3 complex can also be activated by other mechanisms. The active Arp2/3 complex can bind an actin monomer and initiate the nucleation of a new filament at an angle of ~70° to the mother filament. This branched actin-filament network, called the dendritic brush, generates the force needed for cell protrusion at the leading edge (Figure 3). To maintain proper stiffness and maintain the best angle for membrane expansion, the filaments are not allowed to grow very long before their barbed ends are capped by capping protein. The branching of filaments is inhibited by the binding of at least some forms of tropomyosin, which prevent Arp2/3 binding, and branching is also inhibited by proteins in the Ena/VASP family. The binding of tropomyosin and Ena/VASP proteins also inhibits binding of some barbed-end capping proteins, allowing filaments to grow into long parallel bundles, such as those that form filopodia.

## Evolutionary and Structural Relationships between Some Severing and Severing/ Barbed-End-Capping Proteins

The present sequence, structural, and biochemical data suggest that many of the actin-binding proteins have evolved through gene duplication or mutation of DNA sequences encoding a small number of protein motifs. Variants of a single 15 kDa molecular module have evolved, which interact with both G-actin and F-actin with severing and capping functions that may be regulated by calcium and/or by specific phospholipids. Proteins in the ADF/cofilin family are single domain, bind both G-actin and F-actin, have pH-dependent actin-filament severing activity, and are found in all eukaryotes. Twinfilin is a 40 kDa protein with two ADF-homology

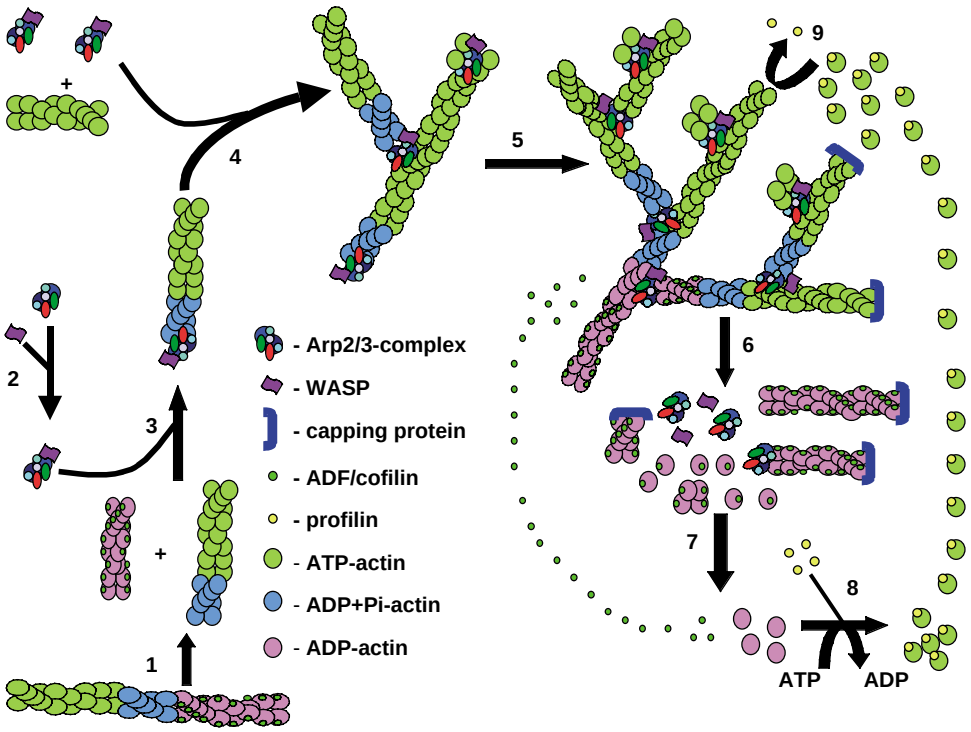



**FIGURE 3** Schematic of actin-filament dynamics at the leading edge of a cell. (1) Severing of actin filament is one mechanism to create new barbed ends. (2) Activation of Arp2/3 complex by WASP can cause *de novo* nucleation of filaments, but active Arp2/3 complex may also be captured by pointed ends of severed filaments (3). (4) Arp2/3 complex either nucleates from the side or ends of actin filaments or when bound to actin, is captured along the side of a filament to create the 70° branches. Further growth of barbed ends with rapid capping and nucleation of new branches leads to complex networks of actin filaments (5). (6) ADF/cofilin-mediated severing and depolymerization of the filament arrays occurs when subunits become ADP-actin. Release of ADF/cofilin (7) and enhanced nucleotide exchange by profilin (8) provide profilin–actin complex that serves as a monomer pool for filament growth (9).

(ADF-H) domains connected by a small linker region. Twinfilin is found across the phyla. Unlike ADF/cofilin, twinfilin does not interact with F-actin and does not promote filament disassembly, but acts as an actin monomer sequestering protein thereby preventing spontaneous actin-filament assembly. CapG, severin, and fragmin contain three structural repeats of the 15 kDa ADF-H domain, and have barbed-end-capping and -severing activity. Presumed duplication of the three repeat-containing proteins gives rise to proteins with six ADF-H repeats, such as gelsolin, villin, and adseverin/scinderin, which are more complicated regulators of actin dynamics. Functions of all of the members of the three repeat and six repeat families are regulated by calcium and specific membrane phospholipids, and are commonly known as the gelsolin family of actin-filament-capping and -severing proteins. Although ADF/cofilins and some gelsolin family members show little or no sequence homology, structurally they display similar overall folding and secondary/tertiary structural elements suggesting both divergent and convergent evolutionary mechanisms may have given rise to these structurally related molecules.

## The Three Segment Proteins: CapG, Severin, and Fragmin

CapG, severin, and fragmin are gelsolin family proteins containing three ADF-H segments. The first segment (S1) binds to G-actin and has the capping function whereas segments 2 and 3 (S2, S3) bind to F-actin. CapG is a 39 kDa barbed-end-capping protein, the name reflecting its capping function and structural similarity to gelsolin, though only half its size. CapG is highly expressed in macrophages and was initially known as macrophage-capping protein, gCap39 or Mbh1. CapG has a structure similar to severin and fragmin, but does not sever actin filaments as does severin and fragmin. CapG caps barbed ends of actin filaments at micromolar concentrations of $Ca^{2+}$, but it dissociates from barbed ends when $Ca^{2+}$ levels return to their normal nanomolar levels, whereas gelsolin remains bound to the barbed ends under the same conditions. CapG is important for the actin-based motility of macrophages. Phosphatidylinositol-4,5-bisphosphate ($PIP_2$) binds to CapG thereby inhibiting its binding to F-actin. The actin monomer sequestering protein profilin is also able to dissociate CapG from actin-filament barbed ends when profilin is present at high concentration ($>10\ \mu M$).

CapG is also found within the nucleus as well as the cytoplasm, but its function there is unknown. Although CapG lacks a nuclear export signal, which is present in severin and fragmin, CapG does not contain any known nuclear localization signal. Thus, the method by which it is translocated into the nucleus is also unknown. Severin and fragmin, found in the slime moulds *Dictyostelium* and *Physarum* respectively, are structurally similar to CapG, but they also bind to the side of and sever F-actin as well as cap the barbed ends of the severed filaments. They have two $Ca^{2+}$-binding sites and can be thought of as small versions of gelsolin.

## The Six Segment Proteins: Gelsolin, Villin, and Adseverin/Scinderin

Gelsolin, villin, and adseverin/scinderin contain six ADF-H repeats and are F-actin barbed-end-capping and -severing proteins. The N-terminal half of these proteins, with three repeats similar to CapG, severin, and fragmin, is able to bind two actin monomers and sever actin filaments. The C-terminal half is able to bind to a single actin monomer at elevated $Ca^{2+}$ concentration.

Gelsolin, with a mass of 80 kDa, is expressed in all eukaryotes. The name arose from its ability to rapidly dissolve an actin gel and convert it into a soluble form. The N-terminal half of gelsolin contains two $Ca^{2+}$-binding sites, one of which is high affinity, in the submicromolar range. Gelsolin severs actin filaments at $Ca^{2+}$ concentrations $>10^{-5}$ M. The severing mechanism probably involves the binding of gelsolin along the side of the filament and intercalation between actin subunits during the normal bending of the actin. After severing filaments, gelsolin remains bound to the barbed end of one new filament (Figure 4) and produces a new pointed end. At $Ca^{2+}$ concentrations in the submicromolar range, gelsolin acts to cap barbed ends of actin filaments. An extracellular (secreted) isoform of gelsolin with molecular mass 83 kDa, called serum gelsolin, circulates in blood where it severs and caps actin filaments released from lysed cells. The resulting monomers of actin are then sequestered by vitamin D-binding protein, a potent monomer-binding protein and ultimately removed from circulation by liver. This mechanism prevents accumulation of actin filaments in the blood, which could potentially block circulation in capillaries.

Gelsolin binding to F-actin is inhibited by tropomyosin in two ways. Tropomyosin binding to F-actin induces a cooperative conformational change in actin filaments resulting in dissociation of gelsolin. Secondly, tropomyosin can directly bind gelsolin and thereby dissociate gelsolin from actin–gelsolin complex. Dissociation of gelsolin by tropomyosin from a gelsolin capped actin filament leads to formation of long filaments from smaller gelsolin-actin complexes, even from gelsolin–actin heterodimers, heterotrimers, and heterotetramers. $PIP_2$ can bind directly to gelsolin and dissociate gelsolin

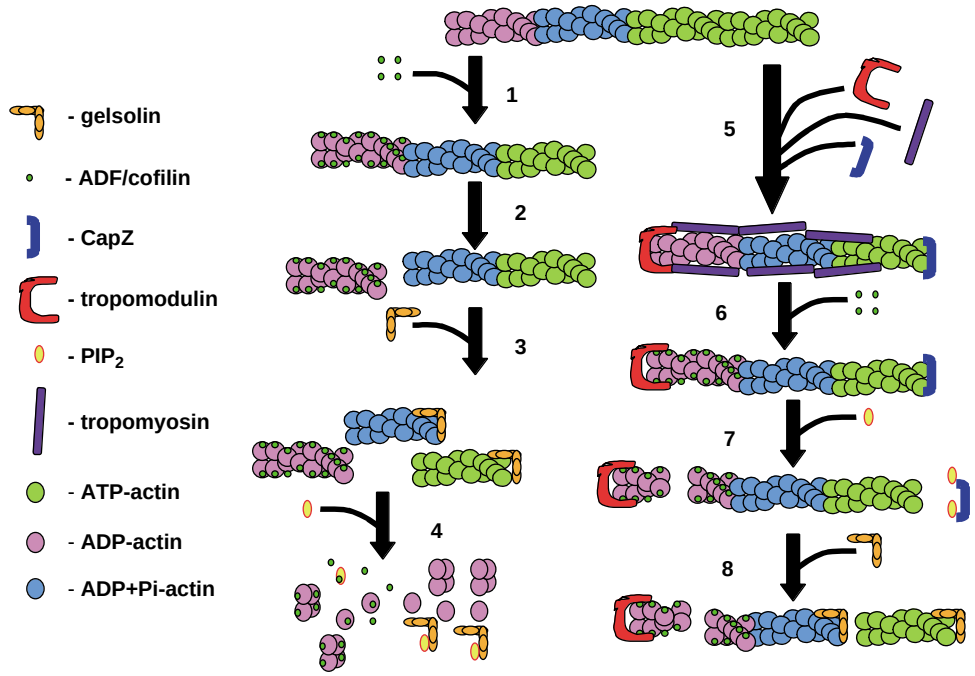



**FIGURE 4** Diagrams showing actin-filament capping and severing by different types of molecules. (1) ADF/cofilin bind with highest affinity and cooperatively to ADP-actin and (2) sever actin filaments as well as enhancing monomer off-rates from the pointed ends (not shown). (5) Stable filaments generated by tropomodulin binding to pointed ends, CapZ binding to barbed ends and tropomyosin binding along filament length. ADF/cofilin competes for F-actin binding with some tropomyosins (6) and can lead to severing and exposure of new pointed ends (7). (4 and 7) $PIP_2$ regulates the binding of capping proteins, dissociating both gelsolin and CapZ from barbed ends of actin filaments. (3 and 8) Actin-filament capping and severing by gelsolin.

that is already bound to the barbed end of an actin filament to yield a free barbed end for growth. Gelsolin can be proteolytically cleaved by caspase-3 during apoptosis. The short amino-terminal cleavage (residues 1–352) fragment does not need $Ca^{2+}$ for severing existing actin filaments and contributing to the progression of apoptosis.

Villin, a gelsolin family member with molecular mass of 92.5 kDa, controls actin assembly in the microvilli of epithelial cells. Villin has the unique property of being able to bundle actin filaments through an 8 kDa F-actin-binding module known as the villin headpiece. The activity of villin is highly regulated by $Ca^{2+}$. At submicromolar $Ca^{2+}$ concentrations, villin bundles actin filaments whereas at micromolar $Ca^{2+}$ concentrations it caps barbed ends of actin filaments and at $>100$ μM $Ca^{2+}$ it severs F-actin. As with gelsolin, the activity of villin is regulated by both $PIP_2$ and $Ca^{2+}$. In addition, villin is regulated by phosphorylation on tyrosine in intestinal epithelial cells, a requirement for regulating the interaction of actin filaments with apical ion channels in the epithelial cells. Deletion of the villin gene in mice leads to accumulation of actin filaments within the epithelial cells making them prone to injury.

Adseverin, also known as Scinderin, is only expressed in secretory cells. It has slightly different temperature and $Ca^{2+}$ sensitivity from gelsolin, but its functions appear to be very similar. During cell stimulation by $Ca^{2+}$, adseverin is activated and disassembles cortical F-actin to allow secretory vesicle exocitosis. As with gelsolin, adseverin binds to and is regulated by $PIP_2$.

## The Single Domain ADF/Cofilin Family

Cell motility and morphogenesis require high rates of turnover of actin filaments, ~100–150-fold faster than occurs for purified actin *in vitro*. The ADF/cofilins are 13–19 kDa proteins containing a single ADF-H domain and are required for this high rate of actin-filament dynamics. They have been isolated or identified by genomic or cDNA analysis from more than 20 different animals, higher plants, fungi, yeasts, and protists. The lethality of the loss of the ADF/cofilin gene in organisms that express only a single gene for this family (e.g., yeast), demonstrates the essential nature of these proteins. Mammals have three separate genes for these proteins: ADF, the ubiquitous cofilin 1 and the muscle-specific cofilin 2. Within any given species, the three proteins are ~70% identical. In an ADF null mouse, cofilin 1 expression is up-regulated to compensate for the loss of ADF and the animals are viable. The only major defect observed is that the mice become blind due to corneal

thickening, even though cofilin 1 is up-regulated in the cornea. This finding, along with differences in the quantitative behavior of the three proteins *in vitro*, suggests that each isoform has some unique tissue specific function. Many ADF/cofilins have nuclear localization sequences, but the protein is usually cytosolic. However, it can be targeted to the nucleus, along with cytoplasmic actin, under certain conditions, although its function there is not known. ADF/cofilins enhance filament turnover (dynamize filaments) in two ways. They can bind cooperatively along the filament, stabilizing a "twisted" form of the actin that is more readily fragmented (severed), creating more filament ends that can either enhance depolymerization, if the environment is an actin monomer sequestering one, or nucleate filament elongation, if the pool of assembly competent monomer is sufficient to sustain growth. If F-actin is severed by ADF/cofilin at the leading edge of cells where active Arp2/3 complex is present, the Arp2/3 complex can capture the pointed ends of the newly severed filaments and bind them along the side of existing filaments to create the highly branched network.

The ADF/cofilin proteins also enhance the rate of subunit loss from the pointed end of filaments. ADF/cofilins bind ADP-actin subunits or monomer with higher affinity than they bind to ATP-actin. Thus, within an actin filament, ADF/cofilin binding is preferentially toward the pointed end, the desired region for filament turnover in cells (Figures 3 and 4). ADF/cofilin activity is regulated by pH: pH $>$ 7.3 favors severing and depolymerization whereas pH $<$ 7.1 favors F-actin binding. ADF/cofilin-mediated F-actin severing is inhibited by tropomyosin, several isoforms of which compete with ADF/cofilin for binding to F-actin. Actin binding, and thus ADF/cofilin activity, is inhibited by phosphorylation at a highly conserved serine residue (ser 3 in mammalian ADF and cofilin). Two related kinases, the LIM kinases and TES kinases, each with two members, phosphorylate ADF/cofilins. The removal of the phosphate from Ser3 may be through the general phosphatases, but a more specific phosphatase called slingshot appears to be the major ADF/cofilin phosphatase for regulated dephosphorylation (activation). Both the kinase and phosphatase pathways are regulated by upstream signaling events through which extracellular signals affect actin dynamics.

Actin-interacting protein 1 (Aip1) is an actin filament-capping protein with a molecular mass of ~65 kDa. *In vitro*, Aip-1 binds to F-actin weakly or not at all in the absence of ADF/cofilin, but caps the barbed end of filaments severed by ADF/cofilin in the presence of ADF/cofilin. Aip1 activity inhibits annealing of the ADF/cofilin severed filaments, maintaining a higher filament number, and blocks the growth at the filament barbed ends. In the nematode *Caenorhabditis elegans*, which expresses two forms of ADF/cofilin, the ubiquitous unc60A and the muscle specific unc60B, only the weaker F-actin-binding/severing species unc60B is able to form a high affinity complex with Aip-1 (unc78). Thus, Aip1 enhances the apparent severing activity of the weaker ADF/cofilin and gives this family of proteins the ability to function much like the severing and capping proteins discussed earlier.

## Other Capping Proteins

There are several capping proteins that appear to be structurally unrelated to those with ADF-H domains. In general these proteins bind weakly to actin monomer but often bind strongly to dimer and thus they can stabilize the dimer and enhance nucleation of filament growth. They have high affinity for the filament ends to which they bind and they usually bind in a 1:1 stoichiometry with the filament (Figures 3 and 4). The main function of capping is to restrict the polymerization/depolymerization, thereby controlling the filament length *in vivo*. As mentioned before, limiting the growth of actin filaments at the leading edge of migrating cells is important for actin-assembly driven membrane protrusion (lamellipodial growth). Pointed-end-capping proteins are important for filament branching, regulating filament turnover, and cross-linking of actin filaments to other components of the cytoskeleton.

### Barbed-End-Capping Proteins

#### CapZ

The most abundant barbed-end-capping protein is CapZ. In striated muscle cells, CapZ binds to actin filaments at the Z-disk, where CapZ also interacts with α-actinin, an actin cross-linking protein, to anchor the thin filaments from the neighboring sarcomere, the contractile unit of skeletal muscle. Nonsarcomeric isoforms of CapZ are localized at the site of membrane actin contact. Cap Z also interacts with two other regulators of actin dynamics, the actin-monomer-binding protein, twinfilin, and with CARMIL, a protein named for its service as a CapZ, Arp2/3 and myosin-I-linker. CapZ is a heterodimer composed of $\alpha$- and $\beta$-subunits, which are 32 and 31 kDa, respectively. Each subunit has two isoforms. Both subunits are structurally similar, and assemble tightly to form a dimer with pseudo twofold rotational symmetry. The C-terminal ends of each subunit are flexible, hydrophobic, mobile in nature, and necessary for actin binding. This flexibility explains how CapZ can tightly bind two actin subunits or the barbed end of the double-stranded F-actin. Cells have capping protein in 1:1 molar ratio with actin filaments and 1:100 to 1:150 with respect to total actin subunits. *In vivo*, the capping activity is regulated by

$PIP_2$, which binds directly to CapZ inhibiting its capping activity. The generation of $PIP_2$ in platelets in response to thrombin, uncaps actin-filament barbed ends allowing localized filament growth needed for the shape changes of platelets associated with blood clot retraction.

### Tensin

Tensin, a large focal adhesion molecule with a mass of ~ 200 kDa, caps the barbed ends of actin filaments within the focal adhesions of fibroblasts and muscle. Tensin interacts with other molecules within the focal adhesion. Tensin is very susceptible to proteolysis, especially by the calcium-activated calpain-II. Full-length recombinant tensin shows complete capping activity whereas "insertin," most likely a proteolytic fragment of tensin, only partially caps F-actin.

## Pointed-End-Capping Proteins

### Tropomodulin

Tropomodulin is a unique pointed-end-capping protein first discovered as an actin-tropomyosin-binding protein (Figure 4). The name tropomodulin was given, because it binds actin filaments tightly in the presence of tropomyosin. Human skeletal muscle tropomodulin is 40.5 kDa, but a related protein named leiomodin is 64 kDa and is found in smooth and cardiac muscle. Tropomodulin caps the pointed end of actin filaments in striated muscle, playing an important role in the assembly of the sarcomere. In striated muscle, tropomodulin is present in a 1:1 molar ratio with respect to actin filaments. The N-terminal half of tropomodulin is rod shaped and binds to the N-terminal part of tropomyosin. The C-terminal half of tropomodulin is responsible for capping the pointed end of F-actin. Although tropomodulin binds to actin filaments tightly, it does not bind to actin monomer. Binding affinity to the pointed end of F-actin is threefold higher in the presence of muscle tropomyosin. Tropomodulin is found in nonmuscle cells and it binds with differing affinities to F-actin containing different isoforms of tropomyosin, suggesting it might play a role in modulating filament turnover through regulating the dynamics at filament pointed ends, where ADF/cofilins function to enhance turnover. However, its cellular role is not well defined.

### Spectrin/Band 4.1

Spectrin/band 4.1 acts as an F-actin pointed-end-capping complex *in vitro*. The proteins are expressed in a wide variety of tissues in multiple isoforms. Spectrin/band 4.1-actin complex was first isolated from red blood cell (erythrocyte) membranes, where actin filaments are short and of uniform length. Both ends of these filaments are capped, the pointed end with spectrin/band 4.1, but the nature of the barbed-end-capping protein is not known. Spectrin binds to some transmembrane proteins as well as to actin, forming a cortical cytoskeleton in erythrocytes.

## See Also the Following Articles

Actin Assembly/Disassembly • Actin-Related Proteins • Rho GTPases and Actin Cytoskeleton Dynamics

## Glossary

**actin depolymerizing factor (ADF)/cofilin** Related actin-binding proteins that increase the turnover of actin filaments *in vivo* through severing and enhancing the off-rate of subunits from the pointed ends of F-actin. The ADF-H domain is used to build larger versions of related proteins that can sever and cap filaments.

**Arp2/3 complex** A complex of two actin related proteins, Arp2 and Arp3, with five other proteins that can nucleate the growth of new actin filaments and cause branching of filaments at the leading edge of a migrating cell.

**Ena/VASP** Enabled/Vasodilator-stimulated phosphoprotein, a family of actin-binding proteins that bind to but do not cap the growing barbed end of actin filaments, reducing branching and aiding in growth of long filaments.

**F-actin** The dynamic filamentous cytoskeletal structures, composed of subunits of actin monomer (G-actin), that organize the cytoplasm to maintain the shape of eukaryotic cells.

**filament capping** The binding of a protein at the end of an actin filament to stop filament growth or depolymerization.

**filament severing** The process whereby a protein binds along the side of an actin filament and severs the filament into smaller pieces.

**filament treadmilling** The dynamic process in which actin filaments elongate at their barbed (plus) end by addition of subunits and at the same time disassemble at their pointed (minus) end with loss of subunits.

**filopodia** Finger-like protrusions with sensory capability that extend from the cell membrane and contain a bundle of parallel cross-linked actin filaments with uniform polarity.

**lamellipodia** Flat web-shaped membrane protrusions containing highly branched actin filaments, seen at the leading edge of many migrating cells.

**WASP** Wiscott–Aldrich syndrome protein, a family of actin-binding protein involved in actin-filament nucleation.

## Further Reading

Bamburg, J. R. (1999). Proteins of the ADF/cofilin family: Essential regulators of actin dynamics. *Annu. Rev. Cell. Biol.* **15**, 185–230.

Carlier, M. F., Le Clainche, C., Wiesner, S., and Pantaloni, D. (2003). Actin-based motility: From molecules to movement. *Bioessays* **25**, 336–345.

Dos Remedios, C. G., Chhabra, D., Kekic, M., Dedova, I. V., Tsubakihara, M., Berry, D. A., and Nosworthy, N. J. (2003). Actin binding proteins: Regulation of cytoskeletal microfilaments. *Physiol. Rev.* **83**, 433–473.

Kwiatkowski, A. V., Gertler, F. B., and Loureiro, J. J. (2003). Function and regulation of Ena/VASP proteins. *Trends Cell Biol.* **13**, 386–392.

Pollard, T. D., and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112**, 453–465.

Puius, Y. A., Mahoney, N. M., and Almo, S. C. (1998). The modular structure of actin-regulatory proteins. *Curr. Opin. Cell Biol.* **10**, 23–34.

Schafer, D. A., and Cooper, J. A. (1995). Control of actin assembly at filament ends. *Annu. Rev. Cell Dev. Biol.* **11**, 497–518.

## Biography

Dr. James R. Bamburg is a Professor of Biochemistry and Molecular Biology and Director of the Molecular, Cellular and Integrative Neuroscience Program at Colorado State University, Fort Collins, Colorado. His principal research interest is the neuronal cytoskeleton and its regulation by assembly modulatory proteins. He holds a Ph.D. degree from the University of Wisconsin, Madison and did postdoctoral research at Stanford University. He has had visiting scientific appointments at the Laboratory of Molecular Biology, Cambridge, UK; Woods Hole Marine Biology Laboratory, Massachusetts; Cell Biophysics Unit, Kings College, London; University of Otago, Dunedin, New Zealand; University of Sydney, Australia; Ludwig Institute, University of California, San Diego, California. He has authored over 100 papers, most dealing with aspects of actin-filament dynamics in neuronal systems.

Dr. Sankar Maiti received his Ph.D. in 2003 from Punjab University where he performed molecular studies on mechanism of protein folding and protein thermostability. He worked as a research fellow in the Institute of Microbial Technology in Chandigarh, India and is currently a postdoctoral fellow in the laboratory of Dr. Bamburg.

# Actin-Related Proteins

**R. Dyche Mullins**
*University of California, San Francisco, California, USA*

**Actin-related proteins (Arps) are a class of proteins found in all eukaryotes and many species of bacteria and archaea. Arps are defined by their degree of similarity to actin (conventional actin), a ubiquitous, eukaryotic cytoskeletal protein. All Arps are built around a common structural fold. This "actin fold" binds and hydrolyzes ATP. In most Arps, ATP hydrolysis is converted into a conformational change that regulates assembly and/or function of multiprotein complexes. Conventional actin assembles into two-stranded helical filaments that form structural scaffolds used to organize the intracellular space and to drive cell division, shape change, and cell locomotion. Eukaryotic Arps fall into ten groups (Figure 1 and Table I) that can be loosely grouped into two categories: (1) cytoskeleton-associated Arps (Arp1, Arp2, Arp3, and Arp10) that regulate the assembly and function of the actin and microtubule cytoskeletons; and (2) chromatin-associated Arps (Arp4–Arp9) that regulate the structure and function of eukaryotic chromatin. Prokaryotic Arps were discovered only recently and their functions are not well understood. The best-characterized prokaryotic Arps (MreB, Mbl, and the plasmid-encoded protein, ParM) polymerize into filaments required for proper cell shape and DNA segregation. The primary sequences of prokaryotic Arps are highly divergent from those of the eukaryotic Arps but we include them in this discussion based on the similarity of their biochemical activities and cellular functions to conventional actin and the eukaryotic Arps. The presence of Arps in chromatin-remodeling complexes and the importance of prokaryotic Arps in DNA segregation suggest that their role in the handling of DNA is as ancient and well conserved as their more well-known cytoskeletal functions.**

## The Actin Fold

There is little or no conservation of surface-exposed residues among Arps. This reflects the fact that the proteins in this family have different binding partners and cellular functions (Figure 1 and Table 1). All Arps, however, share a common structural fold. The core of the protein is formed from two globular domains (domain I and domain II) connected by a flexible hinge (Figure 2B). The interface between the two domains forms an ATP-binding pocket. Five highly conserved sequences – three ATP-binding loops and two connecting helices – line this pocket and define the "actin fold." These sequences are an adenosine-binding loop (adenosine), two phosphate binding loops (phosphate 1 and phosphate 2), and two connecting domains (connect 1 and connect 2). These sequences are conserved in actin, the Arps, and all other members of the actin superfamily (which includes proteins like hexokinase and HSP70) and they are always arranged in the following order: phosphate 1, connect 1, phosphate 2, adenosine, and connect 2 (Figure 2A). The ATP-binding loops are distributed between the two domains of the protein. Phosphate 1 is located in domain I and both phosphate 2 and adenosine are in domain II (Figure 2B). When all three loops interact with ATP the two domains are tightly associated. Hydrolysis of the gamma phosphate of ATP weakens binding to the phosphate 1 domain and causes the two domains of the protein to swing apart around the hinge formed by connect 1 and connect 2. Complete loss of nucleotide results in further opening of the protein around the hinge. In some members of the actin superfamily the binding of ATP to a nucleotide-free protein causes the domains to rotate by 30° around the hinge region (Figure 2C). The shift from closed to open conformation is used by Arps to regulate their interactions with other proteins.

### Prokaryotic Arps

An actin cytoskeleton is thought to be a prerequisite for the establishment and maintenance of intracellular compartments and organelles and until recently actin was thought to be a strictly eukaryotic protein. Recent work, however, has identified actin-like proteins in prokaryotes. The first two prokaryotic actin-like proteins, MreB and Mbl, were discovered in *B. subtilis*. These proteins have very limited primary sequence similarity to conventional actin but, remarkably, they fold into three-dimensional structures very similar to actin and, like conventional actin, they polymerize into two-stranded filaments in a manner that depends on

 © 2004, Elsevier Inc. All Rights Reserved. 

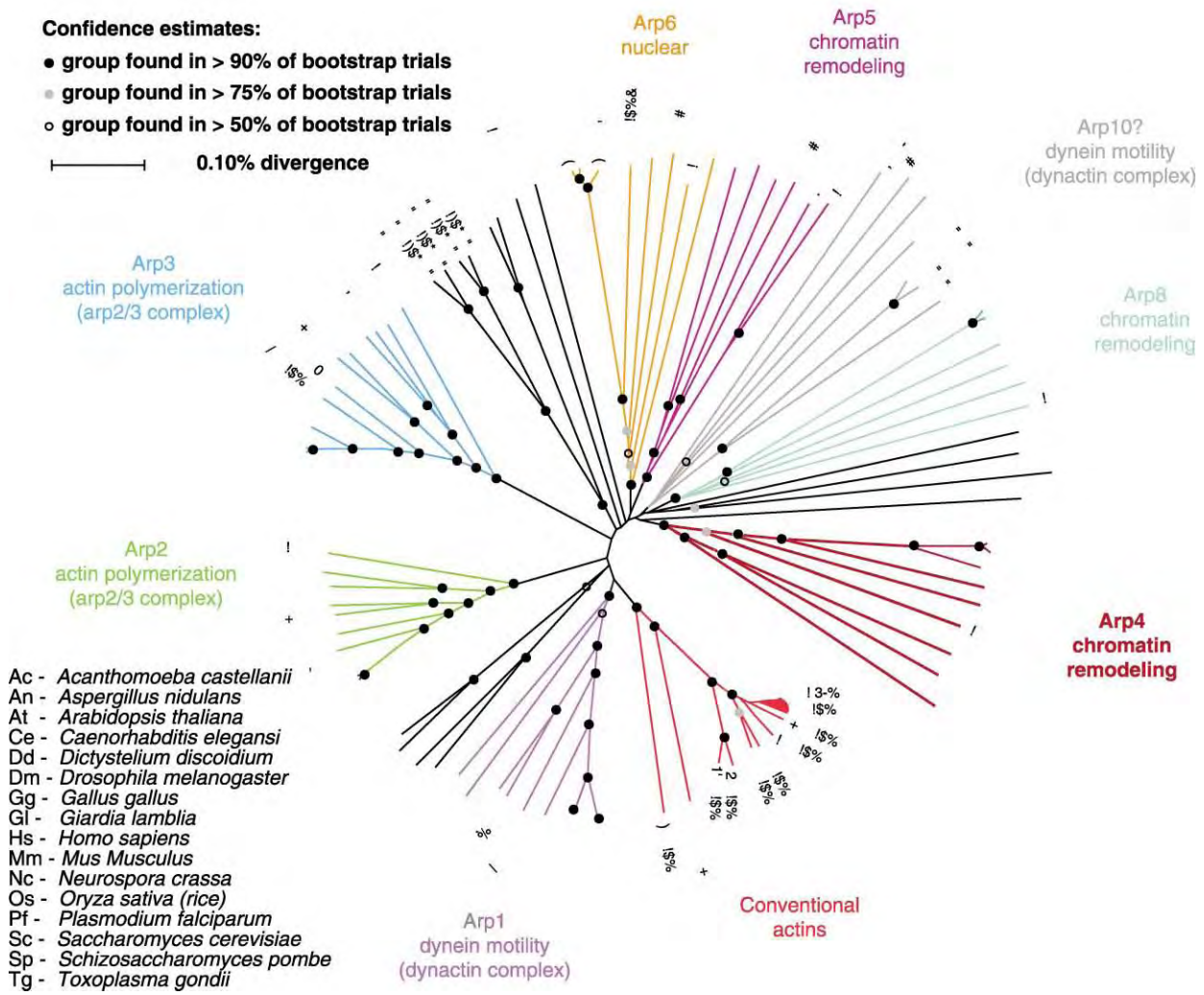



**FIGURE 1** The eukaryotic Arp family is composed of ten, highly conserved members. Members fall broadly into two categories: cytoskeletal Arps (conventional actin, Arp1, Arp2, Arp3, and Arp10) and nuclear Arps (Arp4, Arp5, Arp6, Arp7, Arp8, and Arp9). The diagram is an unrooted phylogenetic tree of the actin superfamily. (Reprinted from *J. Cell Sci.* **202**(115), 2619–2622.) (Calculated by Clustal X Thompson J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucl. Acids Res.* **25**, 4876–4882.

ATP and salt. At present not much is known about the cellular roles of MreB and Mbl, but they do appear to form filaments *in vivo* that are required for proper positioning of the cell-wall synthesis machinery and proper shape determination of rod-shaped bacteria. In addition, MreB and Mbl are required for proper segregation of bacterial DNA during cell division. These are the types of functions performed by polymeric cytoskeletal elements in eukaryotes so it is fair to say that MreB and Mbl form a bona fide bacterial cytoskeleton.

Another remarkable prokaryotic actin-like protein is encoded by the *parM* locus of the low-copy drug resistance plasmid, R100. The R100 plasmid is stably maintained at one copy per cell in bacterial populations because it accurately segregates one copy each into daughter cells during each cell division. Three plasmid loci are required for this accurate segregation: ParR, *parM*, and ParC. ParR encodes a DNA-binding protein that binds tightly to the ParC locus. *parM* encodes an actin-like protein that assembles into two-stranded filaments in an ATP-dependent manner. The ParR protein bound to the ParC locus appears to nucleate formation of *parM* filaments, which drive plasmid segregation. This simple three-piece system may form a structure functionally equivalent to a mitotic spindle and may represent the minimal system for segregating DNA. Unlike conventional actin filaments in which ATP hydrolysis regulates interaction with depolymerizing factors (Figure 3B), ATP binding and hydrolysis directly control assembly and disassembly of ParM filaments. ATP-bound monomers polymerize into filaments.

**TABLE I**

**Cellular Localization, Function and Multi-Protein Complexes of Eukaryotic Arps**

| Arp | Sequence similarity to actin | Cellular localization | Function | Multi-protein complexes |
|---|---|---|---|---|
| Actin | 100% ident. 100% simil. | Cell cortex, leading edge, stress fibers, endosomes, vesicles, nucleus | Cell shape and motility, intracellular trafficking, chromatin remodeling (?), histone acetylation (?) | Actin filaments, INO80, BAF, and NuA4 complexes |
| Arp1 | 45% ident. 68% simil. | Golgi apparatus, transport vesicles, centrosomes, cell cortex | Dynein-driven intracellular movements | Dynactin complex |
| Arp2 | 45% ident. 69% simil. | Cell cortex, leading edge, some motile endosomes | Actin filament nucleation and crosslinking | Arp2/3 complex |
| Arp3 | 39% ident. 60% simil. | Cell cortex, leading edge, some motile endosomes | Actin filament nucleation and crosslinking | Arp2/3 complex |
| Arp4 | 30% ident. 53% simil. | Nucleus | Histone acetylation, chromatin remodeling, control of transcription | NuA4 and INO80 complexes |
| Arp5 | 26% ident. 51% simil. | Nucleus | Chromatin remodeling, control of transcription | INO80 complex |
| Arp6 | 24% ident. 46% simil. | Nucleus | Unknown | Unknown |
| Arp7 | 22% ident. 44% simil. | Nucleus | Chromatin remodeling, control of transcription | Swi/Snf and RSC complexes |
| Arp8 | 21% ident. 44% simil. | Nucleus | Chromatin remodeling, control of transcription | INO8 complex |
| Arp9 | 17% ident. 40% simil. | Nucleus | Chromatin remodeling, control of transcription | Swi/Snf and RSC complexes |
| Arp10 | 17% ident. 38% simil. | Golgi apparatus, transport vesicles, centrosomes, cell cortex | Dynein-driven intracellular movements | Dynactin complex |

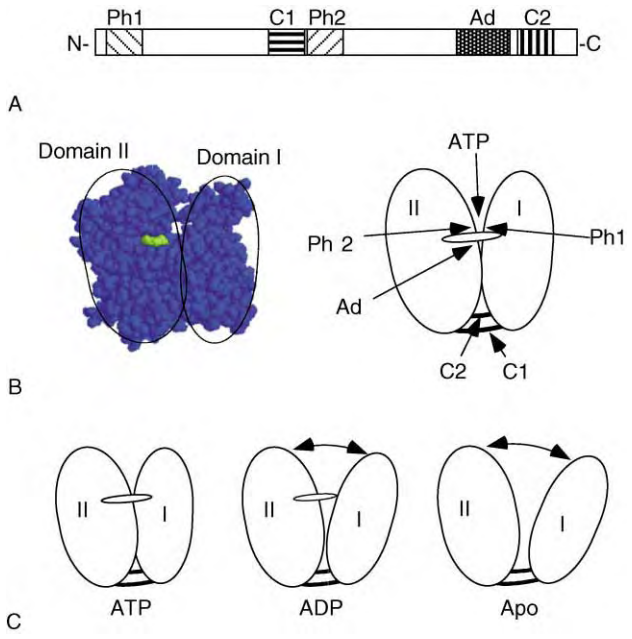



**FIGURE 2** Organization and structure of Arps and the conformational changes driven by ATP binding and hydrolysis. (A) Organization of conserved sequences that define the ATP-binding pocket of the actin fold. Ph1 and Ph2: phosphate-binding loops. C1 and C2: connecting domains. Ad: adenosine-binding domain. The order of these sequences is the same in the primary amino acid sequence of all actin related proteins. (B) Atomic structure and domain organization of an actin monomer. The atomic structure of actin (left) is shown with a bound ATP molecule (green) and with ellipses identifying the two large domains – domain I and domain II – that define the ATP-binding pocket. The phosphate- and adenosine-binding sequences line the interface between domain I and domain II and interact with the ATP molecule (right). The connecting sequences (C1 and C2) link the two protein domains and act as a hinge. (C) Nucleotide-dependent conformational changes in the actin fold. ATP binding holds the domain I and domain II tightly together (ATP, left). Hydrolysis of the $\gamma$-phosphate of ATP to form ADP weakens the interaction with one of the phosphate-binding loops (Ph1) and causes the cleft between the domains to open (ADP, center). Loss of nucleotide from the binding pocket causes the cleft to open even further (Apo, right). Actin-related proteins use this conformational change to regulate their interactions with other proteins.

Within the filament, monomers hydrolyze ATP to ADP. ADP-bound filaments then rapidly disassemble (Figure 3A).

## Eukrayotic Arps

### Cytoskeletal Arps

We will briefly discuss conventional actin, which is well described in a separate article on actin dynamics in this encyclopedia. Arp1 and Arp10 are part of a dynein-activating (dynactin) complex that regulates activity of the microtubule motor protein dynein. Arp2 and Arp3 are subunits of the Arp2/3 complex – a protein complex that nucleates formation of new actin filaments in response to upstream signaling events (see Table I).

## Conventional Actin

Actin is one of the most abundant and highly conserved proteins in eukaryotes. In motile cells actin is typically the most abundant cellular protein and is present at concentrations of 100–200 μM (or 5–10% of total cellular protein). Protozoans, such as budding and fission yeast and slime molds, generally express one isoform of actin. Metazoans typically express multiple, tissue-specific isoforms. Humans express at least six isoforms of actin: two striated muscle isoforms ($\alpha$-skeletal and $\alpha$-cardiac), two smooth muscle isoforms ($\alpha$-smooth muscle and $\alpha$-enteric), and two widely expressed cytoplasmic isoforms ($\beta$-cytoplasmic and $\gamma$-cytoplasmic). The $\alpha$-skeletal and $\alpha$-cardiac striated muscle isoforms are expressed specifically in muscle tissues where they form arrays of "thin" filaments that interdigitate with "thick" filaments composed of the motor protein myosin. Sliding of the thick and thin filaments past each other drives contraction of the muscle tissue. The thin filaments in muscle tissues are very stable structures whose architecture does not change much over time. The smooth muscle actins form less organized filament arrays involved in contraction of vascular and enteric smooth muscle. The cytoplasmic $\beta$- and $\gamma$-isoforms are the most widely expressed and form much more dynamic cytoskeletal structures. The $\beta$-isoform is ubiquitously expressed and is responsible for building the contractile ring that divides cells at the end of mitosis and for most actin-based intracellular traffic. The assembly of $\beta$-cytoplasmic actin also drives cell spreading and the amoeboid, or crawling, motility of many cell types, including those of the nervous and immune systems. The $\beta$-isoform is most closely related to protozoan actins. The $\gamma$-cytoplasmic isoform is expressed in many different cell types but it is much less abundant than $\beta$-cytoplasmic actin. The specific function of $\gamma$-actin is unknown.

The defining property of conventional actin is its self-assembly into helical polymeric filaments. Monomeric actin binds ATP with high (nanomolar) affinity but does not hydrolyze it. The transition from monomer to polymer stimulates the hydrolysis of ATP. Hydrolysis is not required for polymerization but rather for depolymerization. Cleavage of the $\gamma$-phosphate and its subsequent dissociation trigger a conformational change that enables binding of actin depolymerizing factors such as ADF or cofilin (Figure 3B).

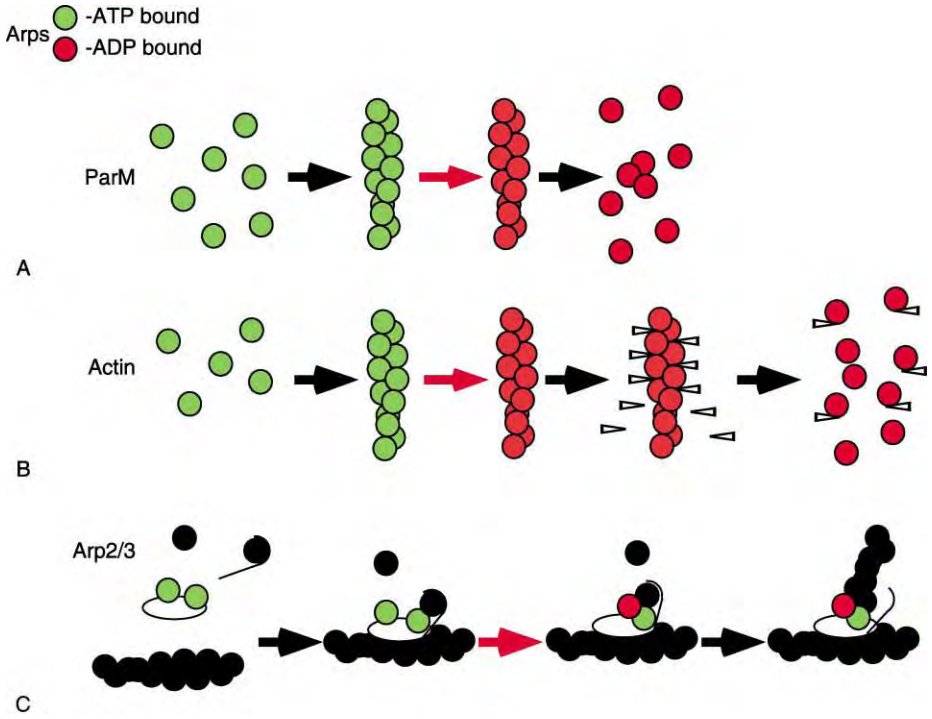



**FIGURE 3** Regulation of actin and Arp function by ATP hydrolysis. The relationship between ATP hydrolysis and protein–protein interactions is best understood in the cytoskeletal Arps. (A) When bound to ATP the plasmid-encoded Arp, ParM (green circles), polymerizes into actin like filaments. Polymerization induces hydrolysis of bound ATP (red arrow); and the ADP ParM filaments (red circles) undergo a conformational change that causes them to rapidly fall apart. (B) Conventional actin and Arp1 (green circles) polymerize into similar two-stranded helical filaments. Polymerization induces ATP hydrolysis on both proteins (red arrow) but the filaments do not spontaneously disassemble. In the case of conventional actin, a conformational change in the ADP filament structure (red circles) promotes binding of actin depolymerization factors (white wedges), which drive filament disassembly. (C) As part of the Arp2/3 complex, Arp2 and Arp3 bind ATP (green circles are ATP-bound Arp2 and Arp3) and nucleate formation of new actin filaments. The nucleation reaction requires monomeric actin and a pre-formed actin filament (black circles) and an Arp2/3 activator (black hook). Assembly of these cofactors induces ATP hydrolysis on Arp2 and promotes a conformational change in the Arp2/3 complex that converts it into a filament nucleus.

## Arp1

Arp1 is most closely related to conventional actin and, like actin, it assembles into a filamentous polymer. Arp1 forms part of a large (1.2 MDa) protein complex that modulates the activity of the microtubule motor protein dynein. Within the dynactin complex, ~10 Arp1 molecules assemble to form a short (37 nm), actin-like filament. This short filament functions as a scaffold that connects dynein motors to cargo vesicles. The Arp1 filaments bind an isoform of the actin-binding protein spectrin that is specific for the Golgi apparatus and for Golgi-derived vesicles. Arp1 and Golgi spectrin are thought to assemble into a membrane-associated meshwork that surrounds vesicles like a cargo net and allows dynein motors to move them around inside the cell.

*In vitro*, purified Arp1 polymerizes into short filaments indistinguishable from conventional actin filaments. Arp1 hydrolyzes ATP upon polymerization but this does not have a dramatic effect on filament stability *in vitro*. Like actin, Arp1 may hydrolyze ATP to modulate its affinity for an accessory factor required for filament disassembly (Figure 3B).

## Arp2 and Arp3

Arp2 and Arp3 are both subunits of a seven-member protein complex, called the Arp2/3 complex that is an essential regulator of the actin cytoskeleton. The Arp2/3 complex nucleates formation of new actin filaments in response to activation of intracellular signaling systems. It also cross-links actin filaments into mechanically rigid networks so that, in general, it functions to convert intracellular signals into cytoskeletal structures. Within the complex the Arp2 and Arp3 subunits are thought to form a heterodimer with a surface that mimics the fast-growing, barbed end of an actin filament. This surface forms a template to initiate

actin filament formation. In the crystal structure of the complex Arp2 and Arp3 are separated by a large (40Å) cleft and formation of an actin-like Arp2–Arp3 dimer would require a significant conformational change. This conformational change may require ATP hydrolysis on one of the Arps (Figure 3C). Binding of ATP to Arp2/3 is required for actin nucleation and neither ADP nor nonhydrolyzable ATP analogues support Arp2/3-dependent actin filament nucleation. Further study is required to understand the connection between ATP hydrolysis and conformational changes on the Arp2/3 complex.

### Chromatin-Associated Arps

Several Arps are associated with eukaryotic chromatin and with chromatin-remodeling complexes. In budding yeast, Arp4, Arp5, Arp7, Arp8, and Arp9 are subunits of ATP-dependent chromatin remodeling complexes. Very little is known about the molecular mechanisms of their activity, but presumably ATPase-driven conformational changes in the Arp subunits regulate the overall structure and activity of these complexes. All of these complexes contain two or four Arp subunits so it is possible that they function as heterodimers and that their interaction is regulated in a manner similar to that of the Arp2/3 complex described earlier.

## Arp4

Arp4 is involved in chromatin remodeling and transcriptional activation. In budding yeast, Arp4 is a subunit of both the NuA4 histone acetyl-transferase complex and the INO80 chromatin-remodeling complex. The NuA4 complex acetylates histones H4 and H2A. Acetylation increases accessibility of the DNA within the nucleosome and recruits other chromatin remodeling enzymes to the region. Human cells contain a similar histone acetyl-transferase complex, called Tip60. This complex also contains an Arp4-type subunit in addition to at least one monomer of conventional actin. The INO80 complex contains Arp4 as well as Arp5, Arp8, and conventional actin. The precise role of Arp4 in these complexes is unknown but yeast Arp4 mutants exhibit phenotypes similar to mutants missing the catalytic acetylase subunit of NuA4, suggesting that it plays an integral role in the function of the NuA4 complex. Arp4 has also been shown to bind directly to histones. Its ATPase activity has not been well studied but Arp4 may use ATP binding and hydrolysis to regulate the interaction between chromatin remodeling complexes and DNA-bound histones.

## Arp5 and Arp8

Arp5 and Arp8 (along with Arp4 and conventional actin) are members of the INO80 chromatin-remodeling complex. INO80 is a large (2 MDa) protein complex that plays a role in transcription of several important yeast genes including pho5, an essential regulator of phosphate metabolism. The total number of yeast genes that require INO80 activity for transcription is not known. Arp8 has been shown to bind directly to histones H3 and H4 so it may be directly involved in moving histones around on the DNA. Arp8 is also required for the incorporation of both Arp4 and conventional actin into the complex so it may play an important structural or scaffolding role.

## Arp7 and Arp9

Arp7 and Arp9 associate tightly to form a heterodimer and dimerization appears to be important to their cellular function. In budding yeast, the Arp7/Arp9 dimer is an essential part of both the Swi/Snf and RSC chromatin remodeling complexes. Like the INO80 complex, these large complexes drive DNA and histone rearrangements necessary for transcription. Swi/Snf activity is required for transcription of ~5% of yeast genes while RSC activity is more globally important. As with the other chromatin-associated Arps, the precise functions of Arp7 and Arp9 are unknown. Loss of the RSC complex is lethal. The loss of either Arp7 or Arp9 causes severe growth defects *in vivo* but does not significantly impair RSC activity *in vitro*. So *in vivo* the Arp7/Arp9 dimer may act to correctly target or regulate the RSC complex.

## Chromatin-Associated Conventional Actin

The large majority of cellular actin is cytoplasmic and generally actin is considered as a cytoskeletal protein whose function is limited to the cytoplasm. In many species, however, chromatin-remodeling complexes contain one or more tightly associated monomers of conventional actin. In addition to Arp4, the NuA4 complex contains a subunit of conventional actin. Also, the yeast INO80 complex and the mammalian BAF complex (a chromatin-remodeling complex similar to the budding yeast swi/snf) contain tightly associated actin monomers. The role of conventional actin in chromatin structure and remodeling is mysterious. It has been speculated that actin assembly in the nucleus could drive large-scale changes in chromatin architecture in the way that actin assembly in the cytoplasm drives

large-scale plasma membrane movements. It is also possible that short actin filaments form an essential scaffold for the assembly and function of chromatin organizing factors.

## Arps of Unknown Function

We know almost nothing about the function of Arp6. In budding yeast Arp6 is localized to the nucleus so it may be part of a chromatin-remodeling complex or it may play a role in determining nuclear architecture.

## See Also the Following Articles

Actin Assembly/Disassembly • Actin-Capping and -Severing Proteins • Chromatin Remodeling • Chromatin: Physical Organization • Nuclear Organization, Chromatin Structure, and Gene Silencing

## Glossary

**actin** Ubiquitous, eukaryotic cytoskeletal protein. Actin monomers assemble into filaments that are used to organize the intracellular space and to define the shape and mechanical properties of eukaryotic cells.

**ATP** Adenosine triphosphate. It is the major energy currency of living cells. The removal or hydrolysis of phosphates from ATP releases energy that individual proteins can use to do work.

**chromatin** DNA that has been packaged and compacted by specialized proteins, called histones. The packaged DNA must be partially unpackaged (by chromatin remodeling complexes) to allow transcription of genes.

**cytoskeleton** A network of protein polymers that organize the intracellular space; transport intracellular cargos; determine cell shape; and drive cell locomotion. The three most important eukaryotic cytoskeletal polymers are actin filaments, microtubules, and intermediate filaments.

**eukaryote** A cell with a nucleus and other membrane-bound organelles.

**histones** DNA packaging proteins. In compacted chromatin DNA is wound around histones like thread around a spool.

**primary sequence** The sequence of amino acids that comprise a protein. Usually given from amino to carboxy terminus.

**prokaryote** A simple cell, such as a bacterium, that lacks a nucleus and other intracellular compartments.

## Further Reading

Bork, P., Sander, C., and Valencia, A. (1992). An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proc. Natl Acad. Sci. USA* **89**, 7290–7294.

Boyer, L. A., and Peterson, C. L. (2000). Actin-related proteins (Arps): Conformational switches for chromatin-remodeling machines? *Bioessays* **22**(7), 666–672.

Carballido-Lopez, R., and Errington, J. (2003). A dynamic bacterial cytoskeleton. *Trends Cell Biol.* **13**(11), 577–583.

Goodson, H. V., and Hawse, W. F. (2002). Molecular evolution of the actin family. *J. Cell Sci.* **115**(Pt 13), 2619–2622.

Herman, I. M. (1993). Actin isoforms. *Curr. Opin. Cell Biol.* **5**(1), 48–55.

Olave, I. A., Reck-Peterson, S. L., and Crabtree, G. R. (2002). Nuclear actin and actin-related proteins in chromatin remodeling. *Annu. Rev. Biochem.* **71**, 755–781.

Pollard, T., Blanchoin, L., and Mullins, R. D. (2000). Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 545–576.

Sterner, D. E., and Berger, S. L. (2000). Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev.* **64**(2), 435–459.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). *Nucl. Acids Res.* **25**, 4876–4882.

## Biography

R. Dyche Mullins is an Assistant Professor of Cellular and Molecular Pharmacology at the University of California Medical School, San Francisco. As an undergraduate, graduate student, and Postdoctoral Fellow he studied Electrical Engineering, Mathematics, and Biochemistry. His research goal is to understand how interactions between signaling molecules and structural molecules determine the architecture of living cells.

# Adenosine Receptors

**Lauren J. Murphree and Joel Linden**
*University of Virginia, Charlottesville, Virginia, USA*

**Adenosine is a purine nucleoside that serves as a link between energy metabolism and cell signaling. Adenosine is a physiologic regulator of all cell types that binds to G protein coupled receptors (GPCRs) that exist as four subtypes ($A_1$, $A_{2A}$, $A_{2B}$, and $A_3$). The receptor-mediated actions of adenosine have been extensively studied in the central nervous system, cardiac muscle, blood vessels, gastrointestinal tract, kidneys, lung, liver, immune system, and other tissues. In light of the clear therapeutic potential of selective adenosine receptor subtype-selective agonists or antagonists, new therapeutic candidates are being intensely pursued by the pharmaceutical industry. Naturally occurring xanthines are nonselective antagonists of adenosine receptors. One such compound, caffeine, is the most widely used psychotropic drug in the world.**

## Adenosine

### Formation

Adenosine is a purine nucleoside that is formed within cells from the breakdown of adenosine monophosphate (AMP), which, in turn, is formed from adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in the course of utilizing energy for cellular functions. Adenine nucleotides released from nerves, mast cells, platelets, endothelial cells, and dying cells also can be dephosphorylated in the extracellular space by ecto-nucleotidases. Increased energy utilization or hypoxia enhances the cellular formation of AMP, which is dephosphorylated by a 5′-nucleotidase in the rate-limiting step for adenosine production. The signaling molecule cyclic AMP (cAMP) can also be a source of AMP for adenosine production. Under resting conditions, a sizable fraction of adenosine is formed by the hydrolysis of *S*-adenosyl-homocysteine.

### Location

Adenosine is produced in either the intracellular or extracellular spaces and can be transported across cell membranes in either direction by a family of nucleoside transporters that allow for equilibration of adenosine concentrations by facilitated diffusion or sodium-dependent active transport.

### Regulation of Production

Production of adenosine is increased during periods of high metabolic activity or ischemia, when there is increased cellular demand for ATP or decreased delivery of oxygen. Adenosine also is derived from adenine nucleotides released from nerves, platelets, mast cells, or other cells. In the brain, adenosine release has been observed experimentally following various manipulations (e.g., hypoxia, ischemia, hypoglycemia, seizures, electrical stimulation, prolonged wakefulness, and application of free radicals). Changes in extracellular levels of adenosine of up to 100-fold have been observed following oxidative or ischemic stress.

### Breakdown of Adenosine

Adenosine is eliminated by metabolic transformation to inosine by adenosine deaminase. It can also be phosphorylated to form AMP by adenosine kinase with a phosphate group donated by ATP, but this enzyme is inhibited in metabolically stressed cells.

## Receptor Structure

Adenosine receptors (ARs) belong to a class of integral membrane proteins known as GPCRs. Members of this class of proteins have seven membrane-spanning $\alpha$-helices with an extracellular amino terminus and an intracellular carboxy-terminal tail. All four subtypes are asparagine-linked glycoproteins and all but the $A_{2A}$ receptor have palmitoylation sites near the C terminus.

### Sequence Similarity

In the mammalian species thus far investigated, differences in protein sequence between species for each receptor subtype are generally much smaller than the differences between subtypes within a given species. For example, cloned mammalian $A_1$ receptors are 90–95% identical, but rat adenosine receptors have

 © 2004, Elsevier Inc. All Rights Reserved. 

similarities of ~60–80% across subtypes. The $A_3$ receptors are an exception to this rule, as they vary by as much as 30% across mammalian species.

### Gene Structure

All four subtypes of adenosine receptors appear to have a similar genomic structure involving two coding exons separated by a single intron located in the region corresponding to the second intracellular loop (between transmembrane regions 3 and 4). The $A_1$ receptor, which is the most well studied, can be transcribed from two different and mutually exclusive promoters. Transcription from these promoters, located approximately 600 bp apart, appears to be differentially regulated. Table I lists some properties of the four AR subtypes.

### Protein Structure

The most precise structural information for proteins has been obtained from X-ray diffraction of protein crystals. GPCRs, like most integral membrane proteins, have proven to be difficult to crystallize due in part to their large size and association with membrane lipids. Thus, most structural information about adenosine receptors has been derived through comparison with the known structures of the related proteins bacteriorhodopsin and mammalian rhodopsin. By comparing the amino acid sequence of an adenosine receptor with the structure of mammalian rhodopsin, researchers have developed models that aid in understanding receptor–ligand interactions. When combined with studies with mutated receptors and pharmacological data, the sites of interactions between the receptors and ligands appear to occur in helices 3, 5, 6, and 7.

## Receptor Physiology and Localization

Adenosine receptor subtypes are differentially distributed throughout the body, allowing for adenosine to initiate a wide variety of physiologic responses. The $A_1$ and $A_{2A}$ ARs have proven to be the easiest to study because of the available pharmacological tools (e.g., radioligands) and their high affinity for adenosine. Consequently, much more is known about the location and physiology of these receptors than about those of the $A_{2B}$ and $A_3$ receptors, which were more recently discovered and have a lower affinity for the natural ligand.

### $A_1$ Receptors

Adenosine acting through $A_1$ ARs has a diverse array of physiological actions. The $A_1$ AR is widely distributed (it is found in, e.g., liver, kidney, adipose tissue, lung, pancreas, and testis) but has been most extensively studied as a regulator of the central nervous system (CNS) and the cardiovascular system.

#### $A_1$ ARs in the CNS

In the CNS, $A_1$ ARs are located in many brain regions (especially the cortex, cerebellum, and hippocampus) and in the dorsal horn of the spinal cord. These receptors are important for regulating sleep and the excitability of neurons. The concept of adenosine as a regulator of sleep follows in part from the fact that caffeine, a naturally occurring AR antagonist, promotes wakefulness. Experimental evidence shows that adenosine concentrations in the brain rise during waking hours but decrease during subsequent recovery sleep. In addition, administration of $A_1$ agonists to brain *in vivo* promotes sleep and reduces arousal. $A_1$ ARs are also important for modulating the excitability of neurons. Adenosine acting at $A_1$ ARs causes an inhibition of synaptic activity by decreasing the neuron's ability to respond to excitatory molecules (i.e., neuropeptides and glutamate).

#### $A_1$ ARs in the Cardiovascular System

$A_1$ ARs are particularly important in the cardiovascular system and are expressed in the heart. Their effects are generally anti-adrenergic, leading to a decrease in heart

**TABLE I**

**Properties of Human Adenosine Receptor Subtypes**

| Adenosine receptor subtype | Length (aa) | Protein Accession No. | GenBank Accession No. | GenBank Accession No. (mouse) | Chromosomal location |
|---|---|---|---|---|---|
| $A_1$ | 326 | P30542 | S45235 | U05671 | 1q32.1 |
| $A_{2A}$ | 410 | P29274 | S46950 | U05672 | 22q11.2 |
| $A_{2B}$ | 328 | P29275 | X68487 | U05673 | 17p11.2–p12 |
| $A_3$ | 318 | P33765 | L22607 | L20331 | 1p13.3 |

rate and blood pressure. One of the most interesting physiological roles of $A_1$ ARs is in the phenomenon of ischemic preconditioning in the heart. During periods of low oxygen supply (ischemia), adenosine is released and activates $A_1$ ARs. This activation begins a cascade of events that protects the heart against a subsequent ischemic event (e.g., infarction and arrhythmia). $A_1$ receptors are also found on sympathetic nerve terminals, where they act to inhibit the release of norepinephrine.

## $A_{2A}$ ARs

$A_{2A}$ ARs are highly expressed by cells in the striatum and olfactory tubercles of the brain, by cells in the spleen and thymus, and by cells in certain blood vessels and immune cells. Lower levels of expression occur in the heart and lung. Like the $A_1$ AR, the most widely studied effects of the $A_{2A}$ AR are in the CNS and the cardiovascular system.

### $A_{2A}$ ARs in the CNS

$A_{2A}$ ARs are highly expressed on small spiny neurons of the striatum, which also express $D_2$ dopamine receptors. The activities of $A_{2A}$ ARs and $D_2$ dopamine receptors are antagonistic. Although $A_{2A}$ receptors are coupled to $G_s$ proteins in many tissues, in the striatum there is evidence to suggest that they signal through the related G protein, $G_{olf}$. Work using mice lacking the $A_{2A}$ AR showed that these receptors also stimulate sensory nerve activity, are involved in mediating pain, and mediate the motor stimulant effects of caffeine.

### $A_{2A}$ ARs in the Cardiovascular System

In the cardiovascular system, the $A_{2A}$ receptor plays two important roles: regulation of blood flow and inhibition of inflammatory cells. Activation of $A_{2A}$ ARs is an important physiological mechanism for coupling energy demand to blood flow. When cells are using large amounts of energy (ATP), they produce adenosine. The adenosine binds to $A_{2A}$ receptors on vascular smooth muscle and causes vasodilation, allowing more blood (and the nutrients it contains) to reach the tissue. $A_{2A}$ ARs also play an autoregulatory role during the process of inflammation. Activation of this receptor causes an array of responses that are generally anti-inflammatory, including inhibition of neutrophil, monocyte, platelet, and T cell activation. $A_{2A}$ ARs also protect against ischemic damage through a mechanism involving its anti-inflammatory properties, which is different from the ischemic preconditioning effects of the $A_1$ AR.

## $A_{2B}$ ARs

The $A_{2B}$ AR is closely related to the $A_{2A}$ AR, though with a much lower affinity for adenosine. This receptor is widely distributed. The epithelium of the cecum, colon, and bladder have high levels of expression of $A_{2B}$. Intermediate levels of expression exist in the lung, blood vessels, and eye, and low levels of expression occur in many tissues (e.g., adipose tissue, adrenal gland, brain, and kidney). The physiological role of the $A_{2B}$ AR is not well characterized, but most researchers agree that it is involved in the relaxation of vascular smooth muscle and in the inhibition of monocyte and macrophage function, effects that are similar to those of the $A_{2A}$ AR. The $A_{2B}$ receptor stimulates fluid and chloride secretion from epithelial cells in the infected gut in response to AMP release from neutrophils, leading to diarrhea. The $A_{2B}$ AR also seems to be involved in the stimulation of mast cell degranulation (a pro-inflammatory event) in some species, including humans.

## $A_3$ ARs

Investigation of the physiology and localization of the $A_3$ AR has been ongoing since the receptor was cloned in 1991 by Meyerhof and co-workers, but characterization has proven to be difficult since there is great variability in its expression and function among species. It is expressed at low levels in the thyroid, brain, liver, kidney, heart, and intestine across species. Rats express high levels in testis and mast cells. In some species (including rats), activation of the $A_3$ AR enhances the release of mast cell mediators. A preconditioning effect similar to that caused by the $A_1$ AR can also result from $A_3$ activation in some species.

# Signaling Downstream of Adenosine Receptors

Adenosine receptors, like all GPCRs, transmit signals to the cell interior by activating intracellular heterotrimeric GTP-binding proteins (G proteins) that consist of $\alpha$-, $\beta$-, and $\gamma$-subunits. Activation of these receptors causes the $\alpha$-subunit to exchange its bound GDP molecule for GTP and become active. The $\alpha$-subunit then dissociates from the $\beta\gamma$ dimer and both can then activate downstream effectors.

The four adenosine receptor subtypes exert their varying effects by coupling to different G proteins, which are summarized in Table II. G protein $\alpha$-subunits control the intracellular levels of many second messengers (e.g., cAMP and inositol triphosphate), which, in turn, activate downstream signaling pathways typical of G proteins. For example, $A_1$ and $A_3$ receptors generally

TABLE II

G Protein Coupling of Adenosine Receptors

| AR Subtype | G protein | Second messengers |
|---|---|---|
| $A_1$ | $G_{i/o}$ | Decrease in cAMP |
| | | Increase in $IP_3$/DAG via PLC |
| | | Activation of PLA2 and PLD |
| $A_{2A}$ | $G_s$ | Increase in cAMP |
| | $G_{olf}$ | Increase in cAMP |
| | $G_{15/16}$ | Increase in $IP_3$ |
| $A_{2B}$ | $G_s$ | Increase in cAMP |
| | $G_{q/11}$ | Increase in $IP_3$/DAG via PLC |
| $A_3$ | $G_i$ | Decrease in cAMP |
| | $G_{q/11}$ | Increase in $IP_3$/DAG via PLC |

*Note*. DAG, diacylglycerol; $IP_3$, inositol triphosphate, PLC, phospholipase C; PLD, phospholipase D.

couple to $G_i$, which inhibits adenylyl cyclase, causing a decrease in intracellular cAMP, whereas $A_{2A}$ and $A_{2B}$ couple to $G_s$, which stimulates cAMP production. In some cells, $A_{2B}$ receptors also are coupled to the calcium-mobilizing G protein, $G_q$.

## Other Ligands

Because of their interesting properties, adenosine receptors are important pharmacological targets. There are several naturally occurring ligands in addition to adenosine, including caffeine, theophylline, and inosine. Inosine is a product of adenosine metabolism, occurs naturally in the body, and can activate $A_3$ receptors at high concentrations that occur in ischemic or inflamed tissues. Caffeine and theophylline are AR antagonists commonly found in coffee and tea.

Many investigators have developed synthetic ligands for adenosine receptors (see Figure 1). It has been particularly challenging to produce ligands that are selective for a given AR subtype, given the high degree of homology between the receptors. Selective compounds are desirable both as tools for understanding receptor functions and as potential drugs. All of the agonist ligands thus far developed are based on the structure of adenosine (an adenine base attached to a ribose moiety). Adenosine is rapidly metabolized. In human, it has a half-life in blood of only 2 s. Modification of this molecule at three different sites has led to agonists with increased longevity, improved selectivity, and nanomolar potency. Some of the most subtype-selective compounds that have been developed are shown in Figure 1.

Caffeine provided a lead compound for the development of xanthine AR antagonists. It belongs to a class of compounds called methylxanthines. Modification of the basic molecule produced high-affinity compounds, some of which show high potency and receptor-subtype selectivity (e.g., MRS1754 in Figure 1). Nonxanthine antagonists (e.g., ZM241385 in Figure 1) were developed later and many appear promising for use in therapeutic applications.

An additional class of ligands for adenosine receptors is known as allosteric enhancers, which were first identified by Bruns and Fergus in 1990. The prototype of the class is PD81723, which binds to the $A_1$ receptor at an allosteric site that is distinct from the adenosine-binding site, known as the orthosteric site. The binding of PD81723 and related allosteric enhancers increases agonist-binding affinity for the $A_1$ AR and amplifies the functional effect of the agonist. Allosteric enhancers seem to stabilize the conformation state of receptors that activate G proteins.

## Therapeutic Possibilities

Adenosine is used clinically to treat certain tachycardias and to dilate coronary arteries to help in the diagnosis of coronary artery disease. There is great interest in developing more selective drugs that selectively activate or block specific adenosine receptor subtypes and that have a longer duration of action than adenosine.

### $A_1$ Receptors

$A_1$ ARs are an attractive pharmacological target because of their ability to simulate ischemic preconditioning. Theoretically, an $A_1$ agonist could protect the heart (and other tissues) from a future ischemic episode, such as myocardial infarction. $A_1$ ARs are also attractive targets for anti-nociceptive agents because of their effects in the CNS. $A_1$ antagonists are undergoing clinical trials as diuretics for use in patients with congestive heart failure. $A_1$ agonists are being developed to reduce heart rate in certain arrhythmias.

### $A_{2A}$ Receptors

Both agonists and antagonists of the $A_{2A}$ AR are being investigated for clinical use. Short-acting $A_{2A}$ agonists may be useful for imaging coronary artery disease since they simulate the effect of exercise and dilate the coronary vasculature. Longer acting $A_{2A}$ agonists may be useful for their anti-inflammatory properties in the treatment of asthma, sepsis, or ischemia–reperfusion injury. $A_{2A}$ antagonists may be helpful in the treatment of CNS diseases, such as Parkinson's disease and schizophrenia.

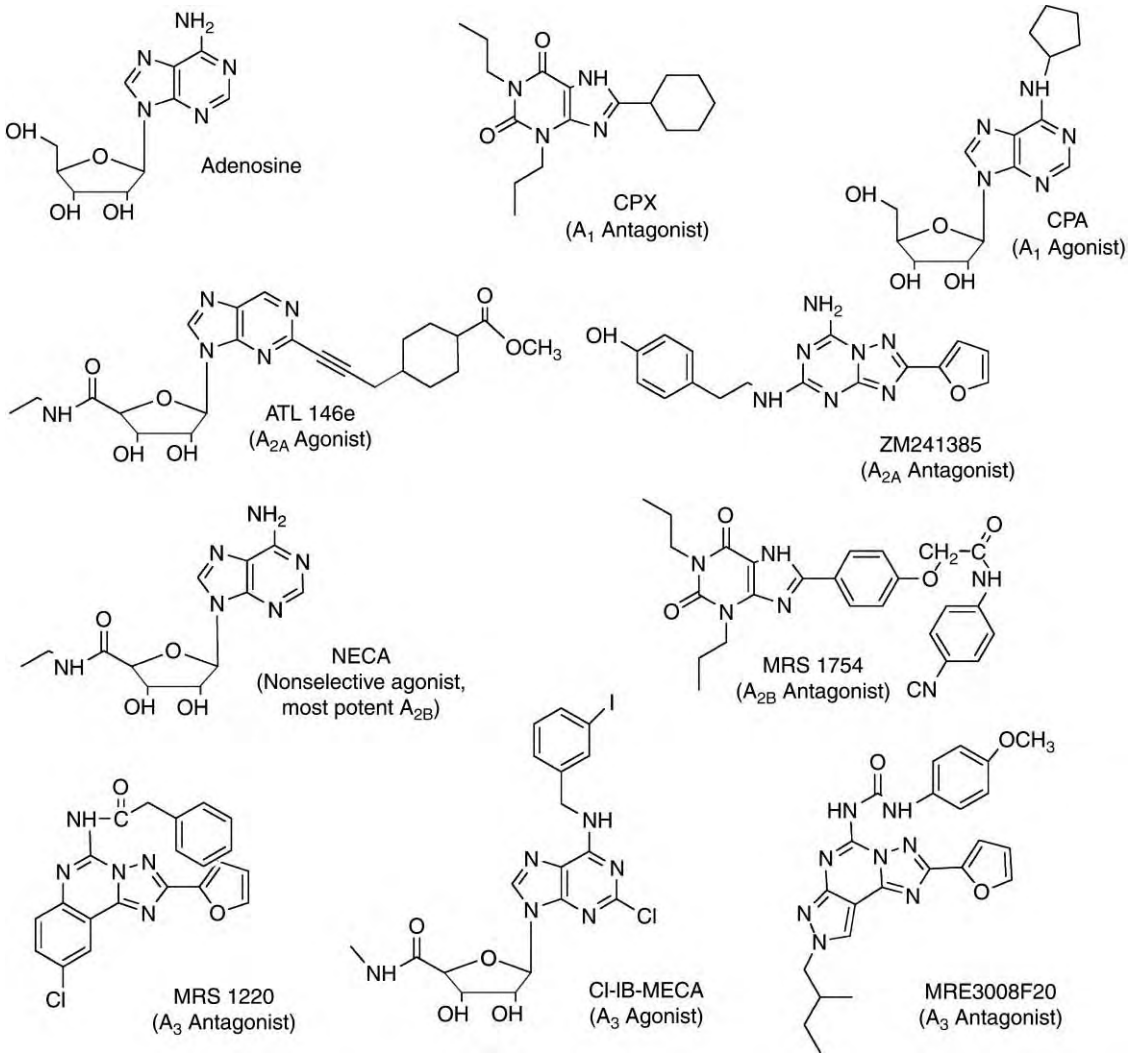



**FIGURE 1** Adenosine receptor ligands.

## $A_{2B}$ Receptors

Antagonists of the $A_{2B}$ AR are being developed as potential treatments for asthma since enprofylline and theophylline are used to prevent bronchoconstriction in asthmatics and are effective at concentrations that would indicate that the $A_{2B}$ AR is its target. There is also some evidence to suggest that $A_{2B}$ blockade may enhance the effects of insulin in liver and skeletal muscle and hence these compounds may be useful for the treatment of diabetes. Blockade of $A_{2B}$ receptors may be useful for inhibiting angiogenesis.

## $A_3$ Receptors

Some researchers have proposed the use of $A_3$ agonists as an adjuvant to chemotherapy since some compounds in this category have an anti-proliferative effect on tumor cells. However, some of these effects appear not to be receptor-mediated.

## See Also the Following Articles

Adenylyl Cyclases • Allosteric Regulation • Diabetes • G Protein-Coupled Receptor Kinases and Arrestins • G Protein Signaling Regulators • Inositol Phosphate Kinases and Phosphatases • Insulin- and Glucagon-Secreting Cells of the Pancreas

## Glossary

**agonist** A ligand that binds to a receptor and causes activation, mimicking the effect of the endogenous ligand.

**antagonist** A ligand that binds to its receptor and inhibits its activity.

**ligand** A chemical or biological substance that binds to a receptor.

**receptor** A recognition site (usually a macromolecule, such as a protein) for a ligand that mediates the ligand's physiological effects.

## Further Reading

Dunwiddie, T. V., and Masino, S. A. (2001). The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci.* **24**, 31–55.

Fredholm, B. B., Arslan, G., Halldner, L., Kull, B., Schulte, G., and Wasserman, W. (2000). Structure and function of adenosine receptors and their genes. *Naunyn-Schmiedeberg Arch. Pharmacol.* **362**, 364–374.

Fredholm, B. B., Izerman, A. P., Jocobson, K. A., Klotz, K. N., and Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* **53**, 527–552.

Klotz, K.-N. (2000). Adenosine receptors and their ligands. *Naunyn-Schmiedeberg Arch. Pharmacol.* **362**, 382–391.

Linden, J. (2001). Molecular approach to adenosine receptors: Receptor-mediated mechanisms of tissue protection. *Annu. Rev. Pharmacol. Toxicol.* **41**, 775–787.

## Biography

Dr. Joel Linden is a Professor of Medicine and Molecular Physiology and Biological Physics at the University of Virginia in Charlottesville. His principal research interests involve the study of adenosine and adenosine receptor signaling. He holds a Ph.D. from the University of Virginia. Dr. Linden is also a consultant to for Adenosine Therapeutics, LLC, which is developing adenosine receptor agonists and antagonists for potential therapeutic use.

# Adenylyl Cyclases

**Ronald Taussig**
*University of Texas Southwestern Medical Center, Dallas, Texas, USA*

**Adenylyl cyclases are enzymes that synthesize the intracellular second messenger, cyclic AMP (cAMP), which in turn triggers a cascade of biochemical changes that regulate a number of important cellular processes. These cAMP-regulated cellular processes play an important role in the control of a number of metabolic processes including the regulation of blood glucose homeostasis, learning and memory, and cell growth.**

## The Hormone-Regulated Adenylyl Cyclase System

In order to function and survive, cells must respond to a multitude of signals present in their environment. These include chemical messengers released from local or distant parts of the body (such as circulating hormones and neurotransmitters) and external stimuli (such as light and odorants). Typically, these chemical messengers do not cross the cell membrane, but rather transduce their signal to the interior of the cell utilizing a "hand-off' mechanism occurring at the plasma membrane. In this process, the stimulus or "first messenger" acts at the membrane to regulate the activity of an enzyme that synthesizes (or degrades) an intracellular "second messenger" that can in turn regulate downstream intracellular metabolic enzymes (Figure 1). Cyclic AMP (cAMP), which is synthesized by adenylyl cyclases, was the first of these second messengers identified, and this mechanism has subsequently shown to be utilized as a common biological strategy to regulate cellular behaviors.

Regulation of intracellular cAMP concentrations is principally controlled at the level of its synthesis, through the hormonal regulation of adenylyl cyclase, the enzyme responsible for the conversion of ATP into cAMP. The adenylyl cyclase system is comprised of three components: heptahelical G protein-coupled receptors for a variety of hormones, neurotransmitters, and odorants; heterotrimeric G proteins; and the catalytic entity itself. Nine isoforms of membrane-bound adenylyl cyclases and a single soluble form have been identified in man. The nine membrane-bound isoforms are subjected to hormonal regulation that is mediated by G proteins of the $G_s$, $G_i$ subclasses. Most importantly, adenylyl cyclase isoforms have the ability to integrate the multitude of hormonal inputs relayed by these pathways to ultimately control intracellular cAMP levels.

## Discovery

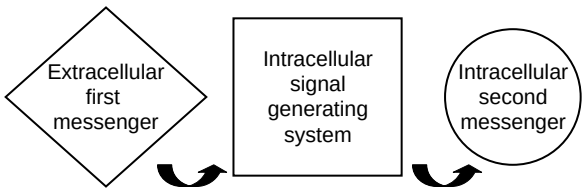
The identification of cAMP resulted from studies aimed at elucidating the biochemical mechanism underlying the breakdown of glycogen in liver cells that is triggered by the hormone glucagon. In his Nobel prize winning discoveries, Earl Sutherland demonstrated that the process involved two distinct stages, a binding of the hormone at the membrane that activated adenylyl cyclase and subsequently generated the second messenger cAMP, and the activation within the cell of the downstream enzymatic process. The later discoveries that the receptor and the adenylyl cyclase were two distinct entities, and the subsequent identification of a third component (the intervening heterotrimeric G protein) have provided our modern day model of the hormone-regulated adenylyl cyclase system. Almost three decades following the discovery of cAMP, the genetic material encoding the first adenylyl cyclase was isolated, which further exposed the complexity of this signaling system.

## Members of the Family

Our current knowledge of the structure and function of the mammalian adenylyl cyclase family has reached a new understanding following the application of molecular approaches. Ten distinct genes encoding the soluble and membrane-bound adenylyl cyclases have been identified (Table I). These genes are broadly distributed on different chromosomes throughout the human genome. Further diversity of adenylyl cyclases is provided by alternatively spliced mRNAs derived from transcripts of some of the family members.

These ten genes are differentially expressed in various tissues in mammalian species. All isoforms of adenylyl

 © 2004, Elsevier Inc. All Rights Reserved. 




| | | |
|---|---|---|
| (a) Adrenaline | Adenylyl cyclase | Cyclic AMP |
| (b) Bradykinin | Phospholipase C | Diacylglycerol |
| (c) Bradykinin | Phospholipase C | Inositol trisphosphate |
| (d) Opiates | Calcium channels | Calcium |
| (e) Light | Phosphodiesterase | Cyclic GMP |

**FIGURE 1** Schematic diagram of second messenger systems. The diagram above the line outlines the progression of cell signaling from the extracellular signal (first messenger) to the generation of intracellular second messengers. The lettered subheadings below the line outline specific examples of intracellular second messengers, their extracellular signals, and the cellular proteins that mediate the changes in the levels of the second messengers. Arrows indicate the direction of the flow of information.

cyclase appear to be expressed in the brain; however, expression of each isoform is limited to specific regions of the central nervous system. Additionally, some adenylyl cyclase appears to be rather widely distributed in most tissues of the body (e.g., types 4, 6, and 7). All adenylyl cyclases have roughly the same size and contain between 1064 and 1610 amino acid residues. The deduced amino acid sequence of all identified adenylyl cyclase isoforms predicts a complex topology within the membrane (Figure 2). The noted exception is the soluble adenylyl cyclase (SAC), which is localized to the intracellular compartment and exhibits regulation quite distinct from the membrane-bound forms. This isoform is more related to an ancestral form found in bacteria than to the other types.

Based on the similarities of their amino acid sequences, and patterns of regulation by G protein pathways, the membrane-bound adenylyl cyclases can be grouped into subfamilies. The first group, comprised of the types 1 and 8, are characterized by their stimulation by calmodulin. The types 2, 4, and 7 adenylyl cyclases form the second group; characteristic of these isoforms is their stimulation by G protein $\beta\gamma$-subunits. Types 5 and 6, which are the two most related isoforms constitute the last group and are characterized by their inhibition by calcium. The type 9 is the largest and most diverse of the adenylyl cyclase isoforms, and like the type 3 enzyme, does not belong to any of the three groups outlined above.

## Regulation of Adenylyl Cyclase Activity by G Proteins and Calcium

There are multiple classes of heterotrimeric G proteins that regulate adenylyl cyclases, either in a stimulatory ($G_s$ family), or inhibitory ($G_i$ family) manner. The two G protein families are normally coupled to distinct receptor subtypes. The $\beta\gamma$-subunits also regulate adenylyl cyclase activity, but in a manner specific to an adenylyl cyclase isoform. Additionally, calcium ions are very strong modulators of some isoforms of adenylyl cyclase; thus, G proteins that regulate calcium entry through voltage-dependent $Ca^{2+}$ channels may also regulate adenylyl cyclase activity.

Hormonal activation of AC occurs primarily through receptors coupled to the stimulatory G protein $G_s$. $G_s$ is the most widely distributed activator of all mammalian membrane-bound AC isoforms. All membrane-bound forms of adenylyl cyclases are regulated by $G_s$ through the interaction of adenylyl cyclase with the G$\alpha$-subunit that is released from the $\beta\gamma$-subunits upon activation by a hormone-bound stimulating receptor (Figure 3A).

Members of the $G_i$ family inhibit adenylyl cyclase activity through the binding of the alpha subunit to the cyclase, but this regulation exhibits selectivity for a

**TABLE I**

**Localization and Expression of the Adenylyl Cyclase Gene Family**

| AC isoform | Length (amino acids) | Location (chromosome) | Sites of expression (tissue) |
|---|---|---|---|
| AC1 | 1135 | 7 | Brain, adrenal medulla |
| AC2 | 1090 | 5 | Brain, skeletal muscle, lung, heart |
| AC3 | 1144 | 2 | Olfactory epithelium, brain |
| AC4 | 1064 | 14 | Widely distributed |
| AC5 | 1184 | 3 | Heart, brain, kidney, liver, lung, uterus |
| AC6 | 1165 | 12 | Ubiquitous |
| AC7 | 1099 | 16 | Ubiquitous (highly expressed in brain) |
| AC8 | 1248 | 8 | Brain, lung, (some in testes, adrenal, uterus, heart) |
| AC9 | 1353 | 16 | Brain, skeletal muscle, lung, heart |
| SAC | 1610 | 1 | Testes |

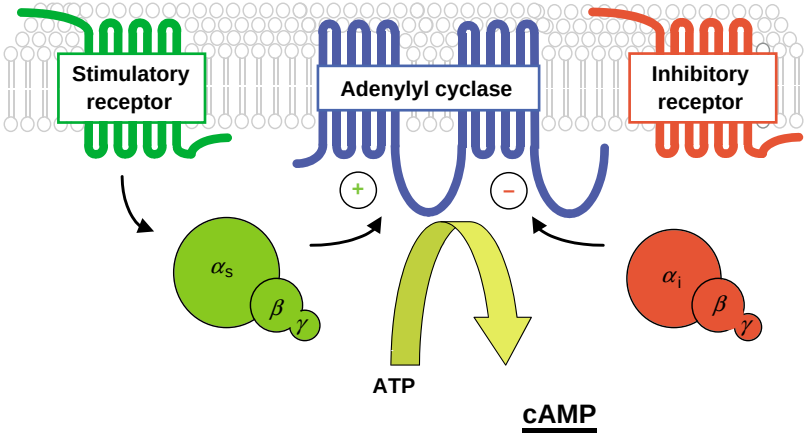



**FIGURE 2** Schematic view of plasma membrane components that participate in the hormone-regulated adenylyl cyclase system. A prototypic adenylyl cyclase is shown. G protein-coupled receptors that enhance or inhibit adenylyl cyclase activity are shown in green and red respectively. All receptors of this family have a similar predicted structure and span the plasma membrane seven times. These receptors couple to their corresponding heterotrimeric G proteins (composed of an $\alpha$-, $\beta$-, and $\gamma$-subunits); $G_s$ (green) and $G_i$ (red) couple to stimulatory and inhibitory receptors respectively. Adenylyl cyclase (shown in blue) is predicted to span the plasma membrane twelve times, and projects its active site towards the cytoplasmic side of the membrane. The result of extracellular messengers binding to the receptors results in the regulation of the conversion of ATP into cAMP by the enzyme adenylyl cyclase.

subset of adenylyl cyclase isoforms (Figure 3B). All three $G_i$ family members ($G_{i1}$, $G_{i2}$, $G_{i3}$) inhibit types 1, 5, 6, and 8; the remaining cyclase isoforms are insensitive to this inhibitory input. Interestingly, this mode of inhibition is not through direct competition with $G_s$ binding to the cyclase, and a number of experimental approaches demonstrate that $G_i\alpha$ exerts its effects at a site, symmetrical to the $G_s$ binding site, and located on the side opposite the cyclase molecule.

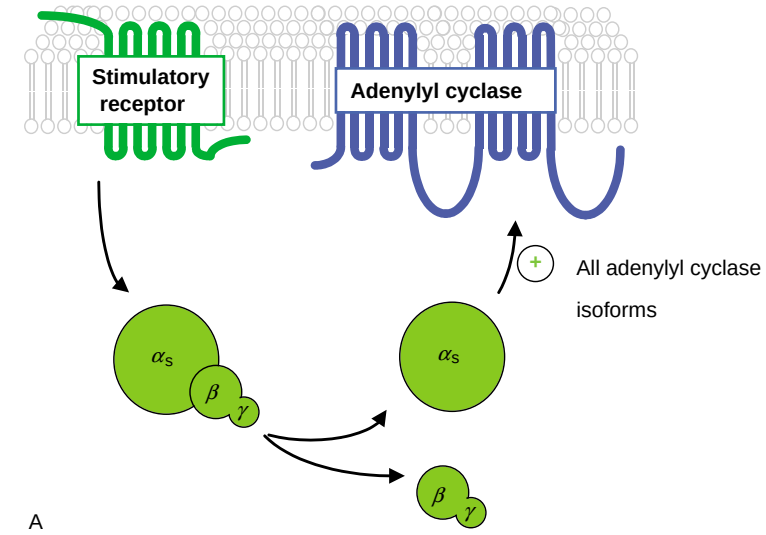
As illustrated in Figure 3C, activation of inhibitory receptors leads to the release of G protein $\beta\gamma$-subunits which can exert profound regulation of adenylyl cyclase activity. Like regulation by the $G_i$ $\alpha$-subunit, this regulation is isoform specific. However, an added complication is that the $\beta\gamma$-subunits can either activate or inhibit the activity – types 2, 4, and 7 are activated whereas types 1 and 8 are inhibited.

Regulation of adenylyl cyclase activity by calcium is still more complex (Figure 4). At physiological intracellular calcium concentrations the types 3, 5, and 6 adenylyl cyclases are inhibited by calcium. For the types 5 and 6, this appears to be due to a direct effect of calcium on the adenylyl cyclase. Stimulation of adenylyl cyclase activity by calcium has been demonstrated for the types 1 and 8 isoforms, but this form of regulation requires calmodulin, a calcium-binding protein that regulates the activity of many cellular enzymes. Indeed, one of these calmodulin-regulated enzymes is a protein kinase, that mediates the inhibitory effects that were observed for the type 3 adenylyl cyclase.

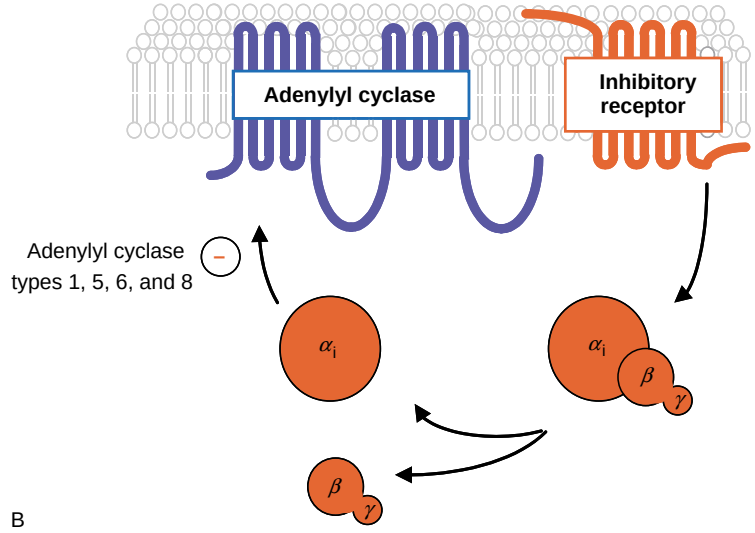
## Physiological Roles of Adenylyl Cyclases

The hormonal activation of adenylyl cyclases and the subsequent regulation of intracellular cAMP impacts on a wide array of cellular processes (Table II). These roles include diverse processes underlying regulation of blood sugar levels, regulation of heart function, water retention as well as higher brain functions of learning and memory. However, while much is known at the biochemical level regarding the regulation of the many adenylyl cyclase isoforms, the precise physiological roles of each isoform are less well known. A further complication is that each cell of our body expresses more than one isoform of adenylyl cyclase. Nevertheless, investigators have begun

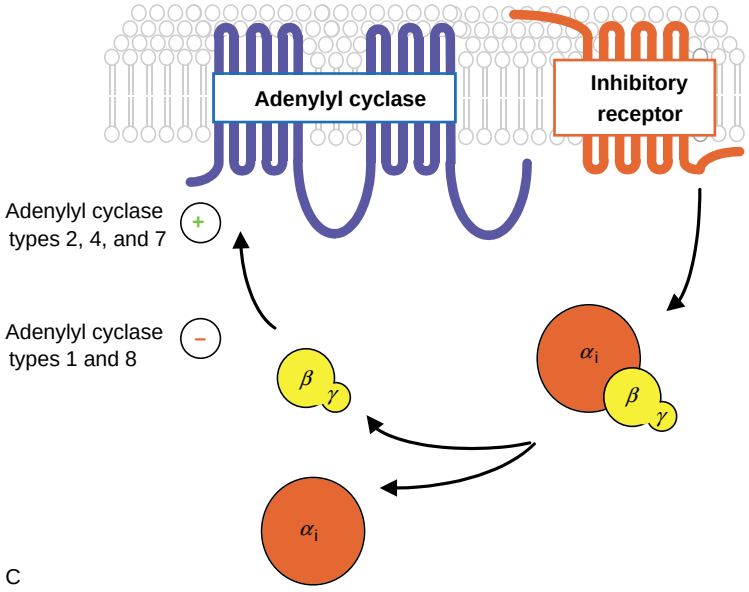
**FIGURE 3** Schematic representation of the isoform-specific regulation of adenylyl cyclases by G protein subunits. (A) Regulation of adenylyl cyclases by $G_s\alpha$. Upon activation of stimulatory receptors, the heterotrimeric G protein, $G_s$ undergoes subunit dissociation whereby the $\beta\gamma$-subunits are released from the $\alpha$-subunit. The $\alpha$-subunit subsequently binds to the adenylyl cyclase and increases its catalytic activity, resulting in an increase in intracellular cAMP levels (+). (B) Regulation of adenylyl cyclases by $G_i\alpha$. Upon activation of inhibitory receptors, the heterotrimeric G protein, $G_i$ undergoes subunit dissociation whereby the $\beta\gamma$-subunits are released from the $\alpha$-subunit. The $\alpha$-subunit subsequently binds to the adenylyl cyclase and decreases its catalytic activity, resulting in a decrease in intracellular cAMP levels (−). Specific isoforms of adenylyl cyclase that are responsive to $G_i$ inhibition are noted. (C) Dual regulation of adenylyl cyclases by G-protein $\beta\gamma$-subunits. $\beta\gamma$-subunits released from $G_i$ heterotrimers can either positively (+) or negatively (−) regulate adenylyl cyclases. Specific isoforms of adenylyl cyclase that are responsive to these 2 types of regulation are noted.


Stimulatory receptor
Adenylyl cyclase
All adenylyl cyclase isoforms
$\alpha_s$
$\beta$
$\gamma$
$\alpha_s$
$\beta$
$\gamma$

A


Adenylyl cyclase
Inhibitory receptor
Adenylyl cyclase types 1, 5, 6, and 8
$\alpha_i$
$\alpha_i$
$\beta$
$\gamma$
$\beta$
$\gamma$

B


Adenylyl cyclase
Inhibitory receptor
Adenylyl cyclase types 2, 4, and 7
Adenylyl cyclase types 1 and 8
$\beta$
$\gamma$
$\alpha_i$
$\beta$
$\gamma$
$\alpha_i$

C

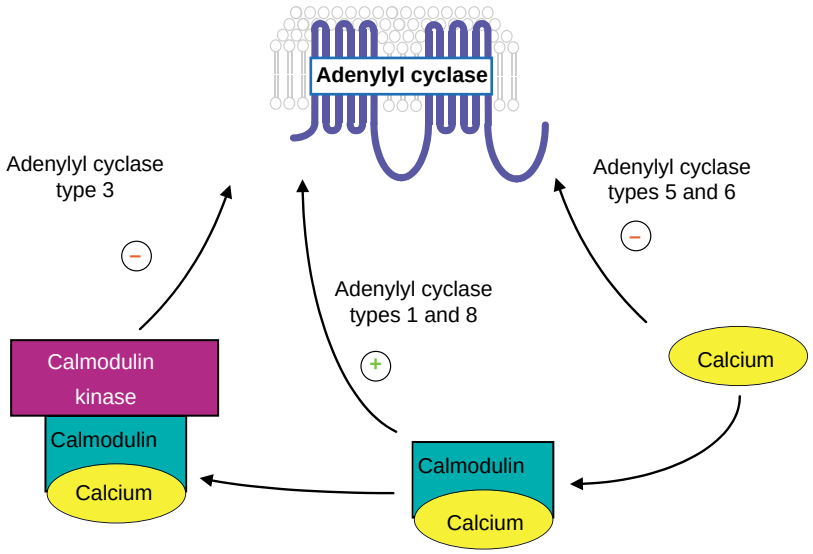



**FIGURE 4** Schematic representation of the isoform-specific regulation of adenylyl cyclases by calcium. Increases in intracellular calcium can regulate the activities of adenylyl cyclases by at least three mechanisms. Calcium can directly bind to and inhibit AC 5 and 6. Calcium bound to calmodulin can bind directly to and activate AC 1 and 8, or can bind and activate a calmodulin-regulated kinase that can subsequently inhibit AC3.

to examine the specific roles of each adenylyl cyclase isoform through the use of mouse strains that are genetically altered by the removal of a particular adenylyl cyclase gene from the mouse genome. Such studies emphasize the involvement of cAMP signaling pathways in pattern formation of the brain and provide definitive evidence for roles of the types 1 and 8 adenylyl cyclases in higher brain function. Similar approaches have revealed a role for the type 3 cyclase in regulation of smooth muscle proliferation in the aorta and recently, a role of the type 5 cyclase in heart function.

A number of studies have uncovered both sporadic and inherited mutations in components of the adenylyl cyclase system associated, or in some cases, shown to be causal to certain human diseases. Mutations causing constitutively active receptors (resulting in elevated intracellular cAMP levels due to constant stimulation of adenylyl cyclase via $G_{s\alpha}$) have been found in patients with familial male precocious puberty/testotoxicosis (LH receptor), hyperfunctioning thyroid adenomas and nonautoimmune autosomal dominant hyperthyroidism (thyroid-stimulating hormone receptor), and Jansen-type metaphyseal chondrodysplasia (parathyroid hormone receptor). Similarly, diseases associated with constitutively activated G proteins ($G_{s\alpha}$) were also identified in patients with endocrine tumors, McCune–Albright syndrome, and testitoxicosis. However, to date no mutations associated with these diseases have been found in the adenylyl cyclase component of this system, but these investigations are ongoing. Here again, a major complication of these studies is the presence of multiple adenylyl cyclase isoforms that are present in each of the cell types.

## See Also the Following Articles

Adenosine Receptors • Adrenergic Receptors • Cyclic GMP Phosphodiesterases • Cyclic Nucleotide

**TABLE II**

**Examples of Physiological Effects Mediated by Adenylyl Cyclases**

| Extracellular signal (hormone or chemical) | Tissue | Isoform (if known) | Effect |
|---|---|---|---|
| Adrenaline | Heart | AC5 and 6 | Increased rate and force of contraction |
| Adrenaline glucagon | Liver | Unknown | Breakdown of glucagon into glucose |
| Neurotransmitters (e.g., serotonin, dopamine) | Brain | Many | Learning, memory, synaptic transmission |
| Chemical odorants | Olfactory epithelium | AC3 | Odorant sensation |
| Antidiuretic hormone | Kidney | Unknown | Regulation of water retention |
| Prostaglandin | Arteries | AC3 | Inhibition of smooth muscle cell proliferation |
| Bicarbonate | Testes | SAC | Fertilization (sperm acrosome reaction) |

Phosphodiesterases • $G_i$ Family of Heterotrimeric G Proteins • $G_s$ Family of Heterotrimeric G Proteins • Phospholipase C

## Glossary

**G protein** A heterotrimeric protein composed of an $\alpha$-, $\beta$-, and $\gamma$-subunit that binds and hydrolyzes GTP and transduces signals from outside to the inside of the cell.

**receptor** A protein that resides in the plasma membrane and specifically recognizes an extracellular signal and upon binding of this signal, transmits information to the cell interior.

**second messenger** A small molecule that is synthesized by a cellular protein and conveys the information within the cell that an extracellular signal has been received.

**signal transduction** The process of transmitting information from the outside to the inside of a cell, resulting in a change in cellular behavior.

## Further Reading

Hanoune, J., and Defer, N. (2001). Regulation and role of adenylyl cyclase isoforms. *Annu. Rev. Pharmacol. Toxicol.* **41**, 145–174.

Johnston, C. A., and Watts, V. J. (2003). Sensitization of adenylate cyclase: A general mechanism of neuroadaptation to persistent activation of G alpha(i/o)-coupled receptors? *Life Sci.* **73**, 2913–2925.

Sunahara, R. K., and Taussig, R. (2002). Isoforms of mammalian adenylyl cyclase: Multiplicities of signaling. *Mol. Interv.* **2**, 168–184.

Tesmer, J. J., and Sprang, S. R. (1998). The structure, catalytic mechanism and regulation of adenylyl cyclase. *Curr. Opin. Struct. Biol.* **8**, 713–719.

Wuttke, M. S., Buck, J., and Levin, L. R. (2001). Bicarbonate-regulated soluble adenylyl cyclase. *JOP* **2**, 154–158.

## Biography

Dr. Ronald Taussig is an Associate Professor in the Department of Pharmacology at the University of Texas Southwestern Medical Center at Dallas. He received a Ph.D. in biology from Stanford University. His principal research interests focus on signaling by heterotrimeric G proteins and the hormone-regulated adenylyl cyclase system. Dr. Taussig is also the Associate Director of the Alliance for Cellular Signaling, a multi-institutional collaboration whose goal is to study and model G protein-mediated and related signaling systems in mouse macrophages.

# Adrenergic Receptors

**David B. Bylund**
*University of Nebraska Medical Center, Omaha, Nebraska, USA*

**Adrenergic receptors (adrenoceptors) mediate the central and peripheral actions of the neurotransmitter norepinephrine (noradrenaline) and the hormone epinephrine (adrenaline); they are widely distributed throughout the body. There are three major adrenergic receptor types: alpha-1, alpha-2, and beta. Each of these three receptor types is further divided into three subtypes. Adrenergic receptors are seven-transmembrane receptors, which consist of a single polypeptide chain with seven hydrophobic regions that are thought to form alpha helical structures that span or transverse the membrane. Because the mechanism of action of adrenergic receptors includes the activation of guanine nucleotide regulatory binding proteins (G proteins), they are also called G protein-coupled receptors.**

## Epinephrine and Norepinephrine

Norepinephrine (noradrenaline) is a neurotransmitter in both the peripheral and central nervous systems. Epinephrine (adrenaline) is a hormone released from the adrenal gland. Norepinephrine and epinephrine are catecholamines, because they have both the catechol moiety (two hydroxyl groups on a benzene ring) and an amine ($NH_2$). Both of these catecholamine messengers play important roles in the regulation of diverse physiological systems by acting through adrenergic receptors. Stimulation of adrenergic receptors by catecholamines released in response to activation of the sympathetic autonomic nervous system results in a variety of effects such as increased heart rate, regulation of vascular tone, and bronchodilatation. In the central nervous system, adrenergic receptors are involved in many functions including memory, learning, alertness, and the response to stress.

Norepinephrine is synthesized starting with the amino acid tyrosine, which is obtained from the diet and can also be synthesized from phenylalanine. Tyrosine is converted to dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase, and DOPA in turn is converted to dopamine. Dopamine is then converted to norepinephrine by the enzyme dopamine beta-hydroxylase. In the adrenal medulla and in a few brain regions, norepinephrine is converted to epinephrine by the enzyme phenylethanolamine N-methyltransferase. The major mechanism by which the effects of norepinephrine are terminated is re-uptake back into the nerve terminal by a high affinity norepinephrine transporter. Epinephrine, as well as norepinephrine are metabolized to inactive products. Norepinephrine is metabolized by the enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) to 3-methoxy-4-hydroxyphenylglycol (MHPG) and 3-methoxy-4-hydroxymandelic acid (VMA). The major metabolite found in the blood and urine is MHPG. Epinephrine is similarly metabolized by MAO and COMT to VMA.

## Classification and Mechanism of Action of Adrenergic Receptors

Adrenergic receptors were originally divided into two major types, alpha and beta, based on their pharmacological characteristics (i.e., rank order potency of agonists). Subsequently, the beta adrenergic receptors were subdivided into beta-1 and beta-2 subtypes; more recently, a beta-3 has been defined. The alpha adrenergic receptors were first subdivided into postsynaptic (alpha-1) and presynaptic (alpha-2) subtypes. After it was realized that not all alpha receptors with alpha-2 pharmacological characteristics were presynaptic, the pharmacological definition was used. The current classification scheme is based on three major types: alpha-1, alpha-2, and beta. Each of these three receptor types is further divided into three subtypes as shown in Figure 1: alpha-1A, alpha-1B, alpha-1D; alpha-2A, alpha-2B, alpha-2C; beta-1, beta-2, beta-3.

The binding of an agonist to an adrenergic receptor induces (or stabilizes) a conformational change that allows the receptor to interact with and activate a G protein. The activated receptor facilitates the exchange of GDP for GTP, leading to the dissociation of the $\alpha$ and $\beta\gamma$_ subunits of the G protein, which in turn stimulate or inhibit the activity various effectors. It is important to note that each of the three types of adrenergic receptors couples to a distinct class

 © 2004, Elsevier Inc. All Rights Reserved. 

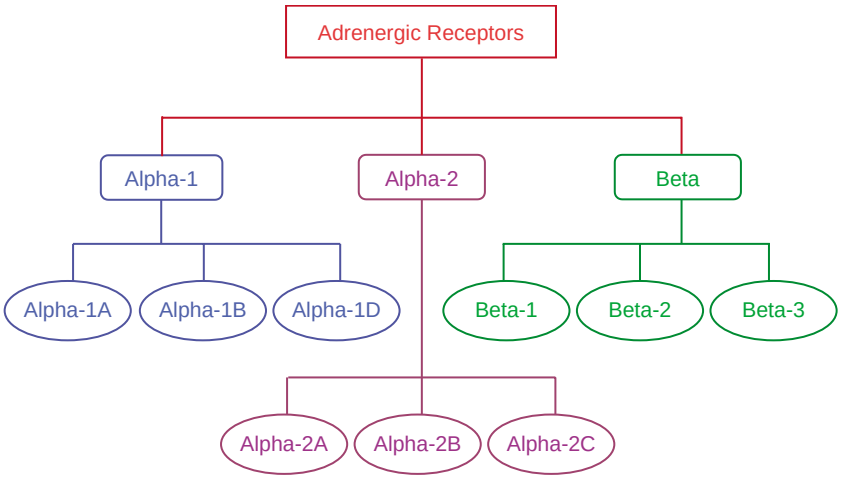



**FIGURE 1** The classification scheme for adrenergic receptors.

of G proteins: alpha-1 to $G_q$; alpha-2 to $G_{i/o}$ and beta to $G_s$. In addition to G proteins, adrenergic receptors interact with other signaling proteins and pathways such as those involving tyrosine kinases.

## Alpha-1 Adrenergic Receptors

Three genetic and four pharmacological alpha-1 adrenergic receptor subtypes have been defined. The alpha-1A and alpha-1B subtypes were initially defined based on their differential affinity for adrenergic agents, such as WB4101 and phentolamine, and on their differential sensitivities to the site-directed alkylating agent chloroethylclonidine. The alpha-1B subtype was subsequently cloned from the hamster and the alpha-1A was cloned from bovine brain, although it was originally called the alpha-1c adrenergic receptor. A third subtype, alpha-1D adrenergic receptor, was subsequently cloned from the rat cerebral cortex, although this clone was originally called the alpha-1a subtype by some investigators. A fourth pharmacological subtype, the alpha-1L, has been identified in vascular tissues from several species, but it may represent a conformational state of the alpha-1A receptor. The current classification scheme includes the alpha-1A, the alpha-1B and the alpha-1D, but there is no alpha-1C (Figure 1).

### Pharmacological and Molecular Characteristics of Alpha-1 Adrenergic Receptors

In addition to norepinephrine and epinephrine, alpha-1 receptors are activated by various agonists such as phenylephrine (Neosynephrine) and methoxamine (Vasoxyl). These agonists are relative selective for alpha-1 receptors and have low affinity for alpha-2 and beta receptors. In contrast, they have similar affinities for the three alpha-1 subtypes and are thus nonsubtype selectively agonists. Similarly, antagonists including prazosin (Minipress) and tamsulosin (Flomax) are relatively selective for alpha-1 receptors and block alpha-2 and beta receptors only at high concentrations. Several other antagonists such as phentolamine (Regitine) and phenoxybenzamine (Dibenzyline) block both alpha-1 and alpha-2 adrenergic receptors with similar affinities. Alpha-1A selective antagonists include 5-methylurapidil and niguldipine, whereas cirazoline appears to be a selective alpha-1A agonist.

The alpha-1 adrenergic receptors are single polypeptide chains of 446 to 572 amino acid residues that span the membrane seven times, with the amino terminal being extracellular and the carboxy terminal intracellular. Thus, there are three intracellular loops and three extracellular loops. In contrast to the alpha-2 receptors, but similar to the beta receptors, the alpha-1 receptors have a long carboxy terminal tail (137–179) amino acid residues) and a short third intracellular loop (68–73 amino acid residues). The amino terminal of the alpha-1A and alpha-1B subtypes have three (alpha-1A) or four (alpha-1B) consensus sites for N-linked glycosylation. The carboxy terminal tails of all three subtypes are potentially palmitolyated, thus anchoring the tail to the membrane and forming a small fourth intracellular loop. The carboxy terminal tails also have multiple sites of phosphorylation that are thought to be important in the desensitization, recycling, and down-regulation of the receptor.

The human alpha-1 adrenergic receptor genes consist of two exons and a single large intron of at least 20 kilobases in the region corresponding to the sixth transmembrane domain. No splice variants are known

for the alpha-1B and alpha-1D subtypes. In contrast, at least 10 splice variants of human alpha-1A subtype have been reported, but only 4 produce full-length receptors.

## Alpha-2 Adrenergic Receptors

Three genetic and four pharmacological alpha-2 adrenergic receptor subtypes have also been defined (Figure 1). The alpha-2A and alpha-2B subtypes were initially defined based on their differential affinity for adrenergic agents such as prazosin and oxymetazoline. These subtypes were subsequently cloned from human, rat, and mouse. A third subtype, alpha-2C, was originally identified in an opossum kidney cell line and has also been cloned from several species. A fourth pharmacological subtype, the alpha-2D, has been identified in the rat, mouse, and cow. This pharmacological subtype is a species orthologue of the human alpha-2A subtype, and thus it is not considered to be a separate genetic subtype.

### Pharmacological and Molecular Characteristics of Alpha-2 Adrenergic Receptors

In addition to norepinephrine and epinephrine, alpha-2 receptors are activated by clonidine (Catapres) and brimonidine (Alphagan). These agonists are relatively selective for alpha-2 receptors and have lower affinity at alpha-1 and beta receptors. Similarly, the antagonist yohimibine is relatively selective for alpha-2 receptors and blocks alpha-1 and beta receptors only at higher concentrations. Antagonists that are at least somewhat selective for one of the alpha-2 subtypes include BRL44408 for the alpha-2A, prazosin and ARC-239 for the alpha-2B (note, however, that these two agents have much higher affinities for alpha-1 receptors), and rauwolscine for the alpha-2C subtype. Ozymetazoline is a partial agonist that has a higher affinity for the alpha-2A subtype when compared to the alpha-2B and alpha-2C subtypes.

The alpha-2 adrenergic receptors are single polypeptide chains of 450 to 462 amino acid residues. In contrast to the alpha-1 and beta receptors, the alpha-2 receptors tend to have long third intracellular loops (148–179 amino acid residues) and a short carboxy terminal tail (20–21 amino acid residues). The amino terminal of the alpha-2A and alpha-2C subtypes have two consensus sites for N-linked glycosylation, and the carboxy terminal tails of all three subtypes are potentially palmitolyated. The third intracellular loops have multiple sites of phosphorylation, which are thought to be important in the desensitization, recycling, and down-regulation of the receptor. The alpha-2 adrenergic receptor genes do not contain introns, and thus there are no splice variants.

## Beta Adrenergic Receptors

Three beta adrenergic receptor subtypes have been identified. The beta-1 adrenergic receptor, the dominant receptor in heart and adipose tissue, is equally sensitive to epinephrine and norepinephrine, whereas the beta-2 adrenergic receptor, responsible for relaxation of vascular, uterine, and airway smooth muscle, is less sensitive to norepinephrine as compared to epinephrine. The beta-3 receptor is insensitive to the commonly used beta-adrenergic receptor antagonists and was previously referred to as the "atypical" beta adrenergic receptor. A beta-4 receptor has been postulated; however, definitive evidence of its existence is lacking, and it is now thought to be a "state" of the beta-1 adrenergic receptor.

### Pharmacological and Molecular Characteristics of Beta Adrenergic Receptors

Isoproterenol (Isuprel) is the prototypic nonsubtype selective beta agonist that has no activity at alpha-1 and alpha-2 receptors except at high concentrations. Epinephrine is 10- to 100-fold more potent at the beta-2 receptor as compared to the beta-1 subtype, whereas norepinephrine is more potent than epinephrine at the beta-3 subtype. Many beta-2 selective agonists, such as terbutaline (Brethine) and salmeterol (Serevent), have been developed for the treatment of asthma. Due to their subtype selectivity, they have a lower incidence of side effects mediated by the beta-1 receptor. Propranolol (Inderal) is the prototypic nonsubtype selective beta antagonist that has equal affinities at the beta-1 and beta-2 subtypes. Other nonselective beta adrenergic antagonists include timolol (Blocadren), pindolol (Visken, which is actually a weak partial agonist), and carvedilol (Coreg), which is also an alpha-1 antagonist. Several beta-1 selective antagonists have been developed, such as metoprolol (Lopressor) and esmolol (Brevibloc).

The beta adrenergic receptors are single polypeptide chains of 408 to 477. In contrast to the alpha-2 receptors, but similar to the alpha-1 receptors, the beta receptors tend to have longer carboxy terminal tails (61–97 amino acid residues) and shorter third intracellular loops (54–80 aa). The amino terminal of the beta receptors have one or two consensus sites for N-linked glycosylation, and the carboxy terminal tails of all three subtypes are potentially palmitoylated. The carboxy terminal tails also have multiple sites of phosphorylation which are thought to be important in

the desensitization, recycling, and down-regulation of the receptor.

The beta-1 and beta-2 adrenergic receptor genes do not contain introns, thus they have no splice variants. In contrast, the beta-3 receptor has one intron, resulting in two splice variants. However, no functional differences have been found between the two splice variants.

## Regulation of Adrenergic Receptors

The processes involved in desensitization and down-regulation have been extensively investigated for the beta-2 adrenergic receptor. The other adrenergic receptors, as well as many other G protein-coupled receptors, appear to behave in a similar manner. Initial uncoupling of the beta-2 receptor from the G protein after agonist binding is mediated by phosphorylation of specific residues in the carboxyl tail of the receptor. The phosphorylated beta-2 receptor serves as a substrate for the binding of $\beta$-arrestin, which not only uncouples the receptor from the signal transduction process but also serves as an adapter protein that mediates the binding of additional signaling proteins and entry into the internalization pathway. The mechanisms of beta-2 adrenergic receptor down-regulation appear to involve both an increase in the rate of degradation of the receptor as well as a decrease in the levels of beta receptor mRNA (8).

## Adrenergic Receptor Signal Transduction Pathways

The alpha-1 adrenergic receptors activate the $G_{q/11}$ family of G proteins leading to the dissociation of the $\alpha$ and $\beta\gamma$ subunits and the subsequent stimulation of the enzyme phospholipase C. This enzyme hydrolyzes phosphatidylinositol 1,2-biphosphate in the membrane producing inositol trisphosphate ($IP_3$) and diacylglycerol. These molecules act as second messengers mediating intracellular $Ca^{++}$ release via the $IP_3$ receptor and activating protein kinase C. Other signaling pathways that have also been shown to be activated by alpha-1 receptors include $Ca^{++}$ influx via voltage-dependent and independent calcium channels, arachidonic acid release, and activation of phospholipase $A_2$, phospholipase D activation, and mitogen-activated protein kinase.

The alpha-2 adrenergic receptors activate the $G_{i/o}$ family of G proteins and alter (classically inhibit) the activity of the enzyme adenylyl cyclase, which in turn, decreases the concentration of the second messenger cyclic AMP. In addition, the stimulation of alpha-2 receptors can regulate several other effector systems including the activation of $K^+$ channels, inhibition or activation of $Ca^{++}$ channels, and activation of phospholipase $A_2$, phospholipase C, and $Na^+/H^+$ exchange.

The beta adrenergic receptors activate the $G_s$ family of G proteins and activate adenylyl cyclase, thus increasing in cyclic AMP concentrations. Beta adrenergic receptors interact with many other signaling proteins, including the phosphoprotein EBP50 (ezrinradixin-moesin-binding phosphoprotein-50), the $Na^+/H^+$ exchanger regulatory factor, and with CNrasGEF.

## Adrenergic Receptor Polymorphisms

Polymorphisms have been identified in some of the alpha-2 and beta adrenergic receptor subtypes, which may have important clinical implications. A common polymorphism has been identified in the third intracellular loop of the alpha-2B receptor, which consists of a deletion of three glutamate residues (301–303); the deletion is a risk factor for acute coronary events, but not hypertension. This deletion results in a loss of short-term agonist-induced desensitization. A common polymorphism has been identified in the third intracellular loop of alpha-2C subtype, which consists of a deletion of four amino acid residues (322–325); the deletion results in an impaired coupling to several effectors.

The gene encoding the human beta-1 adrenergic receptor is quite polymorphic with 18 single nucleotide polymorphisms (SNPs), 7 of which cause amino acid substitutions. A total of 13 polymorphisms in the beta-2 adrenergic receptor gene and its transcriptional regulator upstream peptide have been identified. Three closely linked polymorphisms, two coding region at amino acid positions 16 and 27 and one in the upstream peptide, are common in the general Caucasian population. The glycine-16 receptor exhibits enhanced down-regulation *in vitro* after agonist exposure. In contrast, arginine-16 receptors are more resistant to down-regulation. Some studies have suggested a relationship among these polymorphisms, airway responsiveness (e.g., asthma) and the responsiveness to beta adrenergic agonists.

A tryptophan-64 to arginine polymorphism has been identified in the beta-3 adrenergic receptor. The allele frequency is approximately 30% in the Japanese population, higher in Pima Indians, and lower in Caucasians. Type 2 diabetic patients with this mutation showed a significantly younger onset-age of diabetes and an increased tendency to obesity, hyperinsulinemia, and hypertension.

## See Also the Following Articles

Adenylyl Cyclases • Diabetes • Dopamine Receptors • G Protein-Coupled Receptor Kinases and Arrestins • Inositol Phosphate Kinases and Phosphatases • Phosphatidylinositol Bisphosphate and Trisphosphate • Phospholipase $A_2$ • Phospholipase C • Phospholipase D

## Glossary

**agonist** Compound that binds to a receptor and activates it, thus causing a biological response.

**antagonist** Compound that binds to a receptor but does not activate it. It can block or inhibit the activation caused by an agonist.

**desensitization** Decrease in response of a tissue to a neurotransmitter or agonist drug following repeated administration.

**down-regulation** Decrease in the number or density of receptor binding sites following chronic agonist treatment.

**partial agonist** Agonist that has less than full efficacy in activating its receptor.

**polymorphism** Variability in DNA sequence that occurs with an allele frequency of greater than 1% in the population.

## Further Reading

Bylund, D. B. (1988). Subtypes of $\alpha_2$-adrenoceptors: Pharmacological and molecular biological evidence converge. *Trends Pharmacol. Sci.* **9**, 356–361.

Bylund, D. B. (2003). Norepinephrine. In *Encyclopedia of the Neurological Sciences* (M. J. Aminoff and R. B. Daroff, eds.) Vol. 3, pp. 638–640. Academic Press, San Diego.

Bylund, D. B., Eikenberg, D. C., Hieble, J. P., Langer, S. Z., Lefkowitz, R. J., Minneman, K. P., Molinoff, P. B., Ruffolo, R. R., and Trendelenburg, A. U. (1994). IV. International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.* **46**, 121–136.

Cooper, J. R., Bloom, F. E., and Roth, R. H. (2003). *The Biochemical Basis of Neuropharmacology.* 8th edition, Oxford University Press, New York, pp. 181–223.

Ferguson, S. S. G. (2001). Evolving concepts in G protein-coupled receptor endocytosis: The role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**, 1–24.

Hieble, J. P., Bylund, D. B., Clarke, D. E., Eikenburg, D. C., Langer, S. Z., Lefkowitz, R. J., Minneman, K. P., and Ruffolo, R. R. (1995). International Union of Pharmacology X. Recommendation for nomenclature of alpha-1 adrenoceptors: Consensus update. *Pharmacol. Rev.* **47**, 267–270.

Rockman, H. A., Koch, W. J., and Lefkowitz, R. J. (2002). Seven-transmembrane-spanning receptors and heart function. *Nature* **415**, 206–212.

Small, K. M., McGraw, D. W., and Liggett, S. B. (2003). Pharmacology and physiology of human adrenergic receptor polymorphisms. *Annu. Rev. Pharmacol. Toxicol.* **43**, 381–411.

## Biography

David B. Bylund is a professor and former chair of the Department of Pharmacology at the University of Nebraska Medical Center in Omaha, Nebraska. His main research interests are related to the regulation of adrenergic receptors and their role in mental disorders. He received his Ph.D. from the University of California at Davis and was a postdoctoral fellow at Johns Hopkins University. He was President of the American Society for Pharmacology and Experimental Therapeutics for 2003–2004.

# Affinity Chromatography

**Pedro Cuatrecasas**
*University of California, San Diego, California, USA*

**Meir Wilchek**
*Weizmann Institute of Science, Rehovot, Israel*

**Affinity chromatography (AC) is a variant of liquid chromatography in which biospecific and reversible interactions between biologically active or structurally unique and complementary molecules are used for the selective extraction, separation, purification, analysis, or tagging of specific macromolecules or cell components from crude biological samples. AC was first introduced as a procedure for purifying enzymes and proteins more than 30 years ago. AC is based on the principles of molecular recognition, and today it is one of the most powerful techniques available for purifying physiologically or structurally interacting proteins. In addition to the multitude of proteins purified by AC, the method has also been an indispensable tool for studying many biological processes, such as the mechanism of action of enzymes and hormones, protein–protein or cell–cell interactions, and others encountered in genetic engineering. The emergence of many related methodologies, which are based on molecular recognition (or biorecognition), has impacted virtually all fields of research in the biological sciences.**

## The Key Steps in AC

The first step involves the preparation of a matrix support (resin) material to which a unique "ligand" (typically a small molecule such as an enzyme inhibitor or substrate) is attached (coupled) irreversibly. This is accomplished by chemical activation of the inert resin followed by the irreversible attachment of the ligand (Figure 1). Such a derivatized resin is then used for purification of a biologically active compound by AC following a three-step process as illustrated in Figure 2. These steps are (1) adsorption or reversible attachment of the protein (or macromolecule) to be purified from the mixture to the resin, (2) thorough washing of this resin, and (3) then elution or removal of the protein from the resin. Generally, the used resin can be thoroughly washed and cleaned (regenerated) and used again for the same purpose.

## Why and Where AC Works

Biological macromolecules such as enzymes, polynucleotides (like DNA and RNA), antibodies, receptors, and structural proteins normally interact with chemically different but highly specific molecules by virtue of conformationally unique active sites (such as in enzymes, for substrate binding and catalysis) or binding recognition sites (as for antigens, hormones, and oligonucleotides). If one of these partners of the interacting pair (e.g., enzyme inhibitor) is immobilized on a polymeric carrier, it can be used to attract and isolate the complementary partner (e.g., the enzyme) by simply passing a cell extract containing the latter through a chromatography column packed with the immobilized molecule. Molecules without appreciable affinity for the immobilized ligand will pass unretarded through the column, whereas those capable of binding to the ligand will be retained (adsorbed). Since this binding on the column is based on reversible interactions, the adsorbed component, which is chemically and physically free in solution, will upon continued passage of buffer through the column eventually also come off the column, depending on the strength of the interaction (i.e., affinity). The affinity will depend on factors such as the intrinsic affinity, the effective concentration of covalently bound ligand, temperature, and the composition of the buffer (e.g., pH and salt concentrations). Since the adsorption process is based on the principles of classical bimolecular interactions, a very important factor is also the concentration of the interacting molecule in the solute. The concentration of this component increases progressively as more sample is run through the column, thus effectively increasing its retention on the matrix support during the process of applying the sample through the column. After washing the column, the desired biomolecule adsorbed to the column can be removed (eluted) by varying the buffer conditions.

The distinguishing feature of AC over all other techniques used in purifying macromolecules is the

 © 2004, Elsevier Inc. All Rights Reserved. 

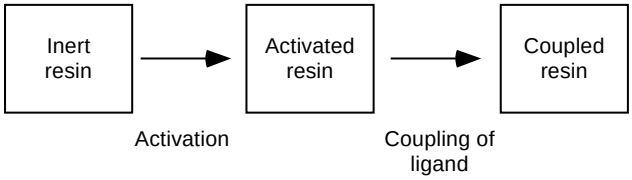



**FIGURE 1** Schematic representation of the process of activation and immobilization of a specific ligand to a surface (resin) for the preparation of specific AC resins (solid supports). For the use of this resin in a purification procedure, see Figure 2.

fundamental dependence on the biological, or functional, rather than on the physicochemical (e.g., size, charge, shape, and hydrophobicity) properties of the molecule to be purified. Virtually all interacting systems consisting of two or more (e.g., when cofactors, metal ions, are essential for complex formation) components are suitable targets for affinity purification. Some of the classes of compounds which have been isolated by AC are described in Table I.

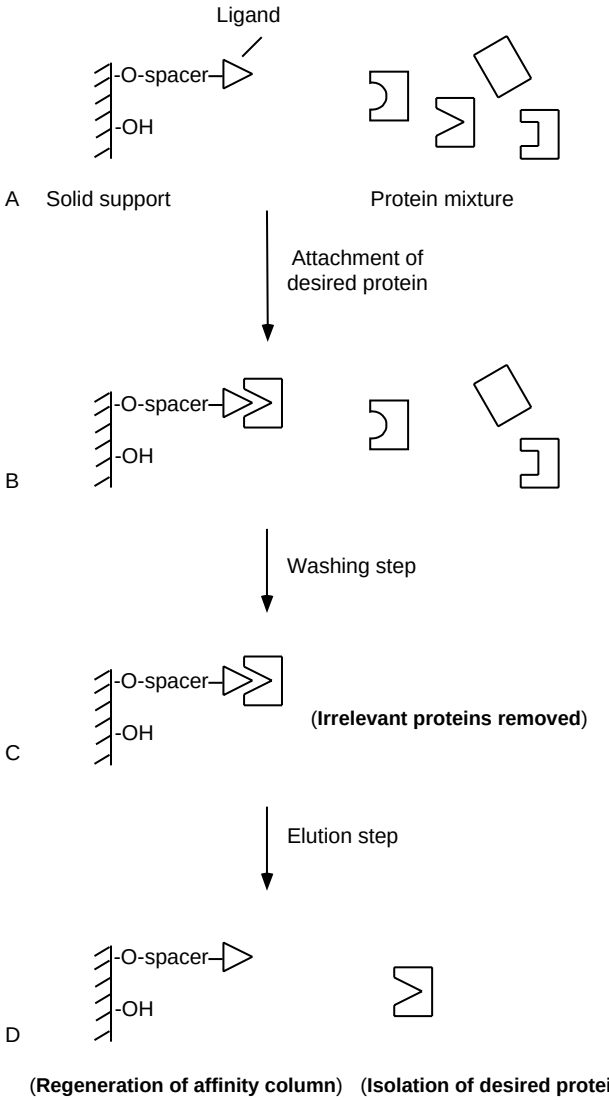



**FIGURE 2** AC involves reversible attachment of a protein to a ligand. (A) The ligand is irreversibly attached to a solid support via a spacer arm; (B) Selective adsorption of a protein from a mixture of proteins; (C) The unadsorbed contaminating proteins are washed away; (D) The desired protein is eluted and recovered from the affinity matrix.

**TABLE I**

**Some Molecular and Cellular Entities Purified by AC**

| |
|---|
| Antibodies and antigens |
| Bacteria |
| Cells |
| Dehydrogenases |
| Enzymes and inhibitors |
| Genetically engineered proteins |
| Hormone-binding proteins |
| Lectins and glycoproteins |
| Receptors (soluble and membrane bound) |
| Regulatory enzymes |
| RNA and DNA (genes) |
| Transaminases |
| Viruses and phages |
| Vitamin-binding proteins |

## The Procedures Used in AC

### Solid Carriers or Matrix Materials Used

Historically, nearly all of the applications of AC were performed under conditions of low pressure, using beaded particles of a size between 50 and 400 μm. A large number of macro-particle support materials for AC are commercially available. By far, the most popular support has been and continues to be agarose. Its success can be attributed not just to its inherently good qualities for AC, but also to its introduction in the initial discovery and its rapid acceptance and widespread use by the research community. Indeed, a literature survey has shown that agarose is used 90% of the time as a solid phase matrix for AC. A number of other supports have also been used successfully in applications of AC. Among these are cross-linked cellulose, trisacryl, Fractogel TSK, and silica (glass beads).

### Activation of and Coupling to Carriers

There are many methods that can be used for the immobilization of ligands to polymeric support materials (carriers). A few of the more representative of these will be described briefly here. The most frequently used method (Figure 3) is the cyanogen bromide activation of agarose that leads to a highly reactive

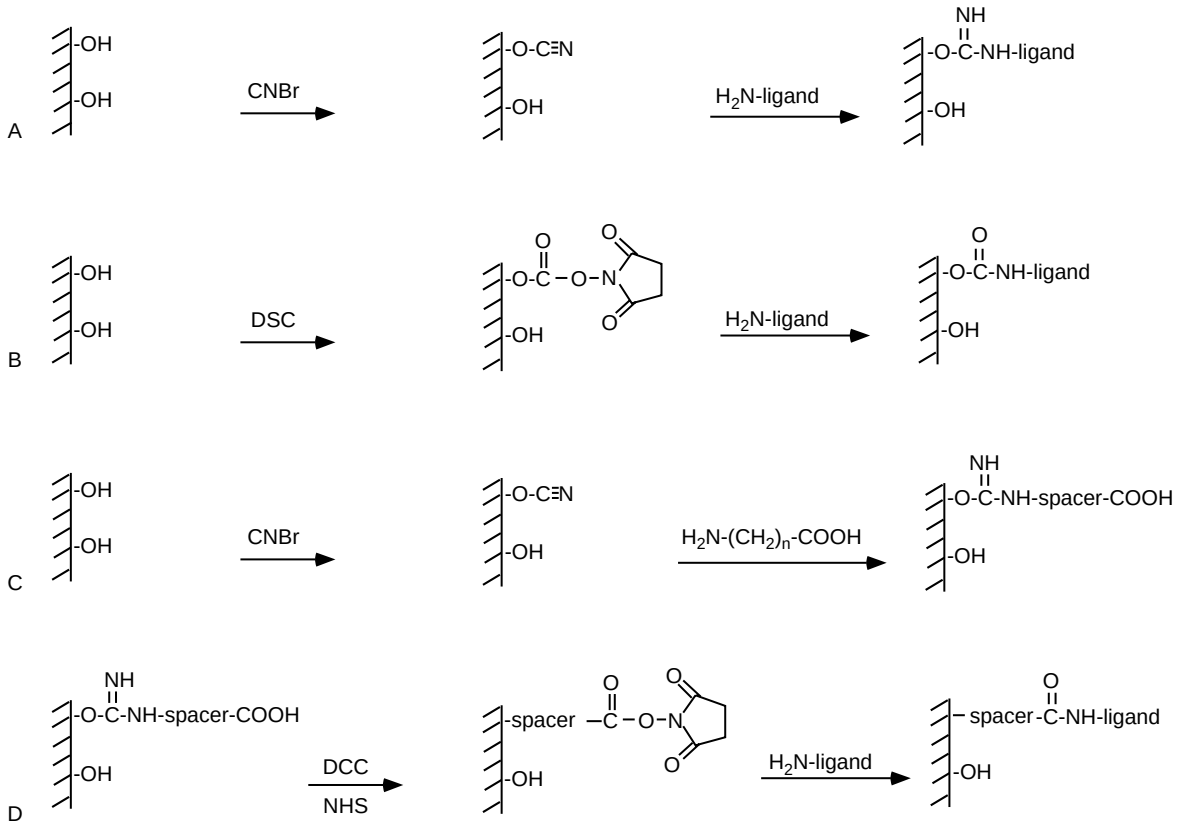



**FIGURE 3** Immobilization of ligands to different activated resins. (A) Cyanate ester, obtained by activation with cyanogen bromide (CNBr), leading to isourea derivative; (B) *N*-hydroxysuccinimide carbonate obtained by activation with *N′N*-disuccinimidyl carbonate (DSC) giving a carbonate derivative; (C) Introduction of a spacer arm using *ω*-amino acids as the spacer resulting in a terminal carboxyl group; (D) Activation of carboxyl groups with dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) yielding a stable amide derivative.

cyanate ester (the activated resin is commercially available). Subsequent coupling of ligands to the activated matrix results in isourea linkages.

Since the earliest days of AC, active esters, in particular *N*-hydroxy-succinimide (NHS) esters, have also been used for immobilizing ligands (Figure 3). The preparation of active esters for subsequent coupling with primary amino group containing ligands requires a matrix that contains carboxylic acid groups. Such matrices can be prepared by activating the hydroxyl groups of agarose with various reagents, including cyanogen bromide and activated carbonates (Figure 3). Successive reaction occurs readily with ligands containing ω-amino acids of various size, depending on the length of the "spacer arm" required. The NHS ester is prepared by mixing the carboxylic matrix with dicyclohexylcarbodiimide and NHS. The covalent attachment via the amino groups of ligands proceeds spontaneously in aqueous solution, resulting in stable amide bonds.

Another method for activating polysaccharide-based polymeric supports like agarose, which contain hydroxyl groups, is the use of *N′N′*-disuccinimidyl carbonate (DSC), which forms highly reactive carbonate derivatives (Figure 3). These derivatives react with nucleophiles under mild, physiological conditions (pH 7.4), and the procedure results in a stable carbamate linkage of the coupled ligand.

## Adsorption

An important step in AC is the selective extraction (adsorption) of the desired macromolecule by its unique binding to the immobilized ligand, thus removing it from the crude mixture in which it was present. The macromolecule, whether an enzyme, antibody, receptor, hormone, or growth factor, is selectively bound to the immobilized specific ligand, which can be a small synthetic or natural molecule, protein, peptide, polynucleotide, nucleotide, polysaccharide, carbohydrate, lipid, or vitamin. Functionally, such ligands may be substrate analogs or inhibitors, antibodies, antigens, coenzymes, or cofactors. The adsorption should be performed under the most favorable conditions for the interaction, including buffer pH, ionic strength, and special ions, as well as temperature. Molecules not possessing appreciable affinity for the immobilized counterpart will pass unretarded through the column.

### Elution

After thoroughly washing the column (usually with adsorption buffer) the desired biomolecule can be eluted by using a buffer containing high concentrations of the same ligand (or a related one, such as a substrate instead of an inhibitor) that was used in the immobilized state. It may be necessary to stop the flow of the column for a period of time, and/or to adjust the temperature, to facilitate the ligand exchange and thus dissociation. Alternatively, the buffer composition can be changed such that the complex will no longer be stable. The buffer conditions usually changed are pH, ionic strength, metal chelators, and organic solvents. The conditions used must not result in irreversible denaturation and inactivation of the eluted macromolecule. If the macromolecule can totally recover its structure and function after total unfolding, effective and complete elution can be achieved with 6 M urea or guanidine (followed by dialysis of the eluate).

## Techniques that Stem from AC

The broad scope of the basic concepts of AC has led to a wide variety of related applications. This has generated various subspecialty adaptations, many of which are now recognized by their own nomenclature, and some of which are covered by their own chapters in this volume. Here, we list some of these (Table II), and briefly discuss a few of the more exciting and recent examples.

**TABLE II**

**Various Techniques Derived from Affinity Chromatography**

| |
|---|
| Affinity capillary electrophoresis |
| Affinity electrophoresis |
| Affinity partitioning |
| Affinity precipitation |
| Affinity repulsion chromatography |
| Affinity tag chromatography |
| Avidin-biotin immobilized system |
| Covalent affinity chromatography |
| Dye-ligand affinity chromatography |
| High performance affinity chromatography |
| Hydrophobic chromatography |
| Immunoaffinity chromatography |
| Lectin affinity chromatography |
| Library-derived affinity ligands |
| Membrane-based affinity chromatography |
| Metal–chelate affinity chromatography |
| Molecular imprinting affinity |
| Perfusion affinity |
| Receptor affinity chromatography |
| Tandem affinity purification (TAP) |
| Thiophilic chromatography |
| Weak affinity chromatography |

### Immunoaffinity Chromatography (IAC)

Among the most popular of these affinity-derived technologies is IAC on antibody columns to purify antigens. The growth of IAC has been ignited by the advancements over the past decade or two in the fields of molecular biology and biotechnology. Great need has existed for purifying pharmacologically active proteins for which ligands of small molecular weight are not available. Also, antibodies can now be produced against virtually any compound. Immobilized antibodies have also been used to remove toxic components from blood by hemoperfusion through affinity resins, and for a variety of applications of solid phase radio- or fluoroimmunoassay. In the biotechnology industry, IAC is providing methods for the large scale preparation of monoclonal antibodies.

### AC and DNA Recombinant Techniques

Recent advances in molecular biology permit determination of the amino acid sequences of proteins by genetic approaches. Thus, large scale isolation of the protein from its native source is often unnecessary, except of course to determine posttranscriptional modifications. Since today most proteins are produced by recombinant technology and genetic engineering, the means for their purification is usually pre-engineered by introducing a biologically active tagging element into the gene itself. Such elements include various temporary "affinity tags" or "affinity tails" such as the His-Tag, which can be purified by metal-chelate AC. FLA–TM-peptide, which consists of eight amino acids, includes both an antigenic site and an enterokinase cleavage site. This affinity tag is used to purify a fusion protein on an immunoaffinity column, and the native protein is recovered by enterokinase cleavage. Fusion proteins can also be produced containing other large proteins such as glutathione transferase, protein A, maltose-binding proteins, cellulose-binding domains, and biotinylated sequences, all of which can serve as targets for purifying the fused proteins on specific ligand affinity columns.

IAC represents a valuable complement, especially when specific ligands that are suitable for immobilization and AC are not available. Today, because of the routine use of recombinant technologies and IAC, pure proteins are so readily obtained that often their function or ligand specificity are the major unknown factors.

We have seen a shift in the challenge toward the "discovery of the ligand," whether the ligand is the naturally occurring species (e.g., what is the substrate, the hormone, or vitamin for this protein), or synthetic. This search can sometimes be achieved using either a phage display library or a combinatorial library to

screen for molecules that may display specific binding. Such a molecule, if found, can then also be used as an affinity ligand for AC. Discovery of ligands can provide insight into the unknown function of genetically coded proteins and serve as useful tools for the purification of proteins or for the discovery of novel medicinal drugs.

### AC and Proteomics: Tandem Affinity Purification (TAP)

In proteomics, the study of protein–protein interactions, especially those occurring in multiprotein cellular complexes, is one of the major challenges. When mass spectrometry (MS) is used, fast and reliable methods of protein purification are necessary. The method perhaps most suitable is affinity purification based on the fusion with tags to the target protein (Tandem Affinity Purification, TAP). The TAP procedures use two different tags for more effective purification on two different affinity columns. These procedures have advantages over other approaches since all of the directly and indirectly interacting components of a protein complex of the expressed protein can be identified and purified in a single experiment. It is claimed that the TAP biphase method will be useful to characterize large, multiprotein complexes, as shown recently in studies of the functional organization of the yeast proteome. In fact, the TAP approach may represent the first real *in vivo* use of affinity purification. This system, however, is not totally without potential problems and it is important to also monitor expression levels to avoid artifacts. Other precautions include the use of immunochemistry to ensure proper cellular localization of the proteins. Another major application of TAP proteins is that they may be used in searching for interacting proteins in discrete cellular domains.

## AC and Biochips

Molecular biosensors based on the affinity concepts described here have evolved over the years in remarkable ways. Recently, a number of technologies have appeared that marry AC between solid phase technologies and sensitive analytic procedures. These include protein and antibody microarrays, mass spectrometric immunoassays, surface-enhanced laser desorption–ionization spectrometry, and surface plasma resonance (SPR). In SPR, a protein, antibody, DNA, or other large macromolecule is attached to a derivatized surface (i.e., glass or metal), creating a biochip. A complex mixture of macromolecules can then be passed over the chip surface and the retained molecules can be detected using various techniques. In some cases, detection is simply a matter of positional location on an ordered array using fluorescent markers; in other cases, the retentates on the chip are desorbed and ionized by lasers. Ionized components, then, are analyzed using time-of-flight or other mass spectrometry techniques. Still in others, mass changes consequent to macromolecular interactions are detected directly using changes in the surface refractive index (by SPR).

The SPR biosensor approach also provides some other unique characteristics. These include (1) access to on- and off-rate analyses of molecular interactions and (2) analyses, in real time, of the interaction dynamics. In addition, SPR has been combined with matrix assisted time-of-flight mass spectrometry to yield a two-dimensional quantitative and qualitative analysis technique. Together, these methods provide a means to detect subtle variations in hundreds of thousands of macromolecules, including single nucleotide polymorphisms in DNA and amino acid changes, or posttranslational modifications of peptides and proteins.

### See Also the Following Articles

Affinity Tags for Protein Purification • Oligosaccharide Analysis by Mass Spectrometry • Recombination-Dependent DNA Replication

### Glossary

**affinity tags** Molecules, like vitamins, peptides, amino acids, proteins, fluorophores or inhibitors, which are incorporated structurally into a protein (often by genetic approaches) to subsequently use with affinity resins to purify (as in AC), or otherwise detect the presence of, or to quantitate, the macromolecule that is thus tagged.

**chromatography** A technique used to separate mixtures of substances on the basis of differences in the ability to adsorb or attach to two different media, one being mobile, which is a moving fluid, and the other being stationary, which is a porous solid or gel, or a liquid coated on a solid support. Substances are carried along by the mobile phase at different rates, depending on their solubility (in a liquid mobile phase) or vapor pressure (in a gas mobile phase) and their avidity for the solid support. Examples are adsorption, column, gas, gas–liquid, gas–solid, gel filtration, high performance liquid, ion exchange, molecular exclusion, partition, thin layer, and affinity (AC).

**ligands** Molecules that bind with specificity and affinity to active sites or special binding sites of macromolecules, as substrates to an enzyme or a hormones to a receptor.

**resin** The solid (stationary) or adsorbent in the chromatographic procedure to which molecules are attached covalently for use in AC. Referred to synonymously as solid support, matrix, polymeric support (insoluble), or carrier.

**spacer arms or extension arms** Linear molecules, like amino acids, diamines or polyethylene glycol (PEG), that are attached irreversibly to the resin and to which specific ligands are then attached covalently for the purpose of allowing macromolecules to interact with the ligand with less potential steric hindrance by the resin.

## Further Reading

Bailon, P., Ehrlich, G. K., Funk, W. J., and Berthold, W. (eds.) (2000). *Affinity Chromatography*. Humana Press, Totowa, NJ.

Cuatrecasas, P. (1972). Affinity chromatography of macromolecules. In *Advances in Enzymology* (A. Meister ed.) Vol. 36, Wiley, New York.

Cuatrecasas, P. (1970). Protein purification by affinity chromatography. Derivatizations of agarose and, polyacrylamide beads. *J. Biol. Chem.* **245**, 3059–3065.

Cuatrecasas, P., and Anfinsen, C. B. (1971). Affinity chromatography. *Ann. Rev. Biochem.* **40**, 259–278.

Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968). Selective enzyme purification by affinity chromatography. *Proc. Natl Acad. Sci.* **61**, 636–643.

Jacoby, W., and Wilchek, M. (eds.) (1974). *Methods in Enzymology*, Vol. 34, Academic Press, New York.

Matejtschuk, P. (ed.) (1997). *Affinity Separations*. Oxford University Press, New York.

Phillips, T. M., and Dickens, B. F. (2000). *Affinity and Immunoaffinity Purification Techniques*. Eaton, Boston.

## Biography

Pedro Cuatrecasas is adjunct professor of Pharmacology and of Medicine at the University of California, San Diego. He received an M.D. from the Washington University School of Medicine, St. Louis. During more than twenty years, he was in charge of research and development at Burroughs Wellcome Co., Glaxo Inc., and Parke Davis/Warner Lambert Co. His research interests have included drug discovery, protein chemistry, hormone action, receptor chemistry and signal transduction.

Meir Wilchek is a professor at the Weizmann Institute of Science in Rehovot, Israel. He received his Ph.D. from the same institute and has been a frequent Visiting Scholar at the National Institute of Health, Bethesda, Maryland. His entire career has been devoted to the study of the biorecognition or "affinity" phenomenon, particularly the application of biorecognition for various purposes.

Cuatrecasas and Wilchek were the first to describe AC, over 30 years ago; they elucidated the basic principles, described the chemical procedures, and explored and suggested applications of these and related technologies.

# Affinity Tags for Protein Purification

**Joseph J. Falke and John A. Corbin**
*University of Colorado, Boulder, Colorado, USA*

**Selective immobilization of proteins greatly facilitates their purification, as well as their biochemical and biophysical characterization. When genetically fused to a target protein, a protein affinity tag provides a powerful tool to selectively capture and immobilize that target, in some cases providing single-step purification. Affinity tags consist of proteins or peptides with distinct amino acid sequences that are capable of a reversible, high affinity binding interaction with a specific partner molecule. The binding partner is linked to a large macroscopic particle or surface that renders it immobile, and thus it is readily amenable to manipulation. The highly specific interaction between the affinity tag and its cognate partner serves as the basis for selective capture of the target protein.**

## Applications of Affinity Tags

Affinity tags have proven to be tremendously effective tools for a wide variety of applications, and they are now incorporated as a standard feature to reduce the number of steps in purification protocols developed for recombinant proteins. In addition, affinity tags have become indispensable in the immobilization of proteins for display on a surface, where the tag ensures that the fusion protein of interest is oriented with its functional regions exposed to docking with other macromolecules. Affinity tags are also used to detect and quantitate target proteins and to analyze protein–protein or protein–ligand interactions. Related technologies are developing rapidly, including bioreactors for multistep enzymatic reactions and bioadsorbants for extraction or degradation of toxic contaminants.

## Construction of a Fusion Protein

An affinity-tagged fusion protein typically consists of a single polypeptide chain with one or more affinity tags coupled to the C- or N-terminus of the target protein or inserted into a loop region. The coupling is via a peptide linker, usually up to 15 amino acids in length. If desired, the linker can contain a specific protease site for affinity tag removal. The use of multiple tags is increasingly common and endows the target protein with two or more unique molecular handles.

### Selection Criteria for an Affinity Tag

Construction of a fusion protein begins with the selection of an affinity tag that is appropriate for the protein of interest (see below). The selection criteria include the size of the affinity tag, the organism that will be used to express the fusion protein, conditions for the immobilization or purification of the fusion protein, and the influence of the affinity tag on the structural and functional characterization of the target protein.

### Affinity-Fusion Gene Constructs

To incorporate the selected affinity tag, a suitable expression plasmid vector is chosen and recombinant DNA techniques are used to insert the target gene next to the affinity tag gene. Numerous vectors are commercially available that code for a selected affinity tag and a linker peptide, the latter often containing an imbedded protease recognition sequence. Additionally, an inducible promoter is present to enhance and regulate fusion protein expression, and a multiple cloning site region is included to facilitate insertion of the target gene with the fusion site at the C- or N-terminus as desired. In some cases, the coding region for a signal peptide is included to promote the secretion of the fusion protein into a specific cellular compartment or into the extracellular medium. Newly developed vectors fuse two different tags to each terminus of a target protein, thereby allowing two-step isolation schemes with enhanced purity and ensuring that only fully intact forms of the fusion protein are isolated.

### Linker Peptide Composition

The composition of the linker peptide can have a substantial impact on the function and utility of the fusion protein; therefore, a number of factors are considered during linker design. Typically, the linker is at least 5–10 residues long to allow free tumbling of

 © 2004, Elsevier Inc. All Rights Reserved. 

the fusion and target proteins relative to one another, thereby preventing unwanted steric hindrance to binding events. For linkers containing a proteolytic cleavage site (typically for enterokinase, factor Xa, thrombin, or tobacco etch virus (TEV)), inclusion of 5–10 flanking amino acids at both ends of the site ensures adequate accessibility to protease. Finally, the linker amino acid composition must be selected to minimize susceptibility to host organism proteases.

## Overview of Fusion Protein Production and Immobilization

Purification of a recombinant fusion protein begins with the introduction of the expression plasmid into the host cell, followed by cell growth and induction of the fusion promoter. The host cells are lysed and the resulting lysate, containing the affinity-tagged protein, is incubated with a solid phase support to which the binding partner is coupled. Following binding of the tag to its immobilized partner, the support is washed to remove unbound components, yielding specific isolation of the tagged protein. If desired, the tagged protein can be released by either disrupting its interaction with the binding partner or by proteolysis of the linker region. Isolation and immobilization procedures often need to be optimized for each new affinity-tagged protein and expression host.

## Features and Types of Affinity Tag Systems

The large number of affinity tags currently available offers a diverse spectrum of biochemical properties that can be exploited for protein immobilization in a variety of contexts. Affinity tags range in size from short peptides less than 1 kDa to proteins as large as 120 kDa, and they can be classified into general categories based on their binding partner interaction such as protein–ligand, polyamino acid–matrix, antigen–antibody, and protein–protein. New types of tags based on novel binding partner interactions continue to emerge at a rapid pace.

When choosing an affinity tag for a specific application, several features are considered. A subset of affinity tags possess the useful property of binding to their partner even under denaturing conditions. The ability to immobilize a tagged protein under denaturing conditions is a great advantage when the fusion protein is expressed in an insoluble or non-native form. Other tags utilize a binding partner interaction that is easily disrupted under mild conditions, thereby facilitating the recovery of target protein with full biological activity. Affinity tag size can also impact target protein function and structural integrity. Small tags generally have less impact on protein structure and function and often need not be removed, while large tags can be perturbing and thus are typically removed prior to structural and functional studies. On the other hand, certain large tags can significantly enhance the solubility of a fusion protein expressed at high levels; therefore, they are appropriate choices for the isolation of poorly soluble target proteins. Finally, when a target protein is toxic to the expression host, affinity tags that promote the aggregation of fusion protein into insoluble aggregates known as inclusion bodies are used. Key features of representative affinity tags are discussed below, and Table I presents a more comprehensive list.

### Protein–Ligand Interaction Affinity Tags

Enzymes and small molecule-binding proteins are designed to bind their ligands with high specificity, thus many highly effective affinity tag systems are based on ligand-binding interactions. One of these is the widely utilized glutathione S-transferase (GST) tag (26 kDa) that binds with high specificity and low affinity ($K_D \sim 180\ \mu M$) to its substrate glutathione. Although the affinity of the GST tag for its ligand is low, commercially available immobilized glutathione matrices provide high local ligand concentrations that ensure adequate retention of the fusion protein. GST-tagged proteins are typically isolated from crude cell lysates by their interaction with immobilized glutathione on beads then eluted by competitive displacement with free, reduced glutathione. The low ligand affinity enables elution under mild, nondenaturing conditions. The GST tag often increases fusion protein solubility and stability, and it is ideally suited for overexpression of recombinant proteins in their native state. Often, the GST tag is proteolytically removed at the end of the purification due to its large size and tendency to form dimers. In other applications, the GST tag is retained and used to couple the target protein to a bead or surface for biochemical or biophysical studies.

Another family of affinity tags that relies on protein–ligand interactions are the cellulose-binding domains (CelBD). CelBDs are small, highly stable protein modules consisting of between 33 and 180 amino acids (3–20 kDa) that bind to different forms of cellulose or chitin with a broad range of affinities ($K_D \sim 0.01$ to $400\ \mu M$). CelBD fusions can be constructed with affinity tags inserted internally or linked to either the C- or N-terminus of the protein of interest. Over 180 different CelBDs have been identified, providing a spectrum of polysaccharide specificities, binding affinities, and targeting properties. Certain CelBDs bind

cellulose essentially irreversibly, making them ideal for protein immobilization, while other CelBDs exhibit easily reversible binding. Denaturing conditions or proteolysis are often needed to elute irreversible CelBDs, while reversible CelBDs can be eluted under mild conditions such as the use of a competitive ligand (cellbiose or ethylene glycol) or desorption with water. Finally, CelBDs can be selected that target the fusion protein for secretion or inclusion body formation.

The 51 amino acid chitin-binding domain (ChiBD) is derived from *Bacillus circulans* chitinase and exhibits high affinity, essentially irreversible binding to its ligand chitin, a natural carbohydrate polymer. ChiBD fusion proteins are immobilized under physiological conditions by adsorption to chitin coupled to a solid phase material. ChiBD fusion proteins often have an intein element incorporated into the linker region in place of a proteolytic recognition sequence. When remobilization of the fusion protein is desired, the intein element is activated and undergoes self-cleavage, resulting in the release of the target protein, while the intein-ChiBD region is retained on the solid phase. Similar inteins will probably soon be incorporated into the linkers of other affinity tags. Several other protein–ligand affinity tag systems are currently in use, each having unique advantages. For example, the 40 kDa maltose binding protein (MalBP) is a large tag that often increases fusion protein solubility. MalBP binds to cross-linked amylose with moderate affinity ($K_D \sim$ micromolar range), permitting MalBP tagged fusion proteins to be immobilized in a reversible fashion. The full length MalBP tag directs a fusion protein to the oxidizing *E. coli* periplasm, where MalBP is a native protein and where the fusion protein may benefit from disulfide bond formation. Alternatively, removal of the MalBP leader peptide yields retention of the fusion protein in the cytosol, where the greater volume can allow higher expression levels.

## Polyamino Acid–Matrix Interaction Affinity Tags

Immobilized-metal affinity chromatography (IMAC) is a common technique used to purify recombinant proteins fused to a short peptide affinity tag. IMAC, which can be carried out under denaturing or nondenaturing conditions, relies on the interaction between multiple electron donors on the affinity tag with a transition metal ion ($Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$) chelated to a solid phase support. The affinity tag is typically polyhistidine, ranging 6 to 12 residues in length fused to the N- or C-terminus of the target, where "6-His" is most common and the electron donor is the histidine imidazole ring. Recently, an affinity tag based on a natural peptide derived from the N-terminus of chicken lactate dehydrogenase has also been utilized. This HAT-tag contains 6 histidine residues interspersed within a 19-residue polypeptide (KDHLI HNVHK EEHAH AHNK) that binds to specifically $Co^{2+}$-carboxymethylaspartate and has a lower net charge than polyhistidine tags. The most widely employed metal chelator is iminodiacetic (IDA), while nitrilotriacetic acid (NTA) and carboxymethylated aspartic acid (trade name TALON) are also popular. The chelator is generally covalently coupled to polymer beads, or ferromagnetic beads for magnetic isolation. Adsorption of IMAC tagged proteins is normally performed at neutral to slightly basic pH to ensure that the histidine imidazole groups are not protonated. Mild elution conditions include ligand exchange with imidazole, extraction of the metal ion by a strong chelator like EDTA, or proteolytic elution. Low pH will also elute, but it can sometimes denature the target protein. IMAC is not recommended for target proteins possessing a metal center, since the metal can be stripped by the binding partner chelators.

The Arg-tag is another example of a small polyamino acid affinity tag, in this case designed to raise the isoelectric point of the fusion protein to enhance its binding to a cation exchange matrix. Mild elution conditions are generally a NaCl gradient at alkaline pH. This tag also binds to flat mica sheets, which may enable a variety of new applications.

## Antibody–Epitope Interaction Affinity Tags

Recombinant DNA technology has been employed to create peptide epitopes that bind well-characterized antibodies. This process is known as epitope tagging and is complementary to a more traditional approach where a novel antibody is generated for an existing epitope. Both approaches have been exploited to develop affinity tag systems based on the binding interaction between a peptide epitope and its specific antibody partner. In some cases, several antibodies with different properties are available for a given epitope. Proteins fused to peptide epitope affinity tags can be captured by immunoaffinity interactions via immobilized monoclonal antibodies. Typical epitope tags range from 6 to 30 residues in length, are highly charged, and have little effect on protein structure–function. They can be fused at either the C- or N-terminus, or even inserted within the protein of interest. Epitope tag expression vectors are commercially available for mammalian, insect, yeast, and bacterial host cells and provide a variety of tags including c-myc, FLAG, HA, T7, V5, VSV-G, recA, Protein C, Protein A, and Protein Z. Some of these are optimized for immobilization while others are primarily used for protein detection. The importance of epitope tags is illustrated by their many applications including analysis of *in vivo* protein expression,

**TABLE I**

**Commonly Used Affinity Tags and Their Features**

| | | | | | | Nondenaturing | | |
|---|---|---|---|---|---|---|---|---|
| **Category of tag** | **Name of tag** | **Number of residues in tag** | **Size of tag (kDa)** | **Location of tag** | **Immobile phase binding partner** | **Immobilization conditions** | **Affinity tag elution conditions (eluent)** | **Expression host[a]** |
| Protein–ligand interactions | Glutathione S-transferase | 211 | 26 | N-Term, C-Term | Glutathione | Yes | Yes (10 mM Reduced Glutathione) | B, Y, I, M |
| | Cellulose-binding domains | 27–189 | 3–20 | N-Term, C-Term, Internal | Cellulose and other cellulosics | Yes | Yes (water) or No (Guanidinium HCL) | B, Y, I, M |
| | Maltose-binding protein | 396 | 40 | N-Term, C-Term | Cross-linked amylose | Yes | Yes (10 mM Maltose) | B |
| | Chitin-binding domain | 51 | 5.6 | N-Term, C-Term | Chitin | Yes | No | B |
| Polyamino acid–matrix interactions | Polyhistidine | 6–12 | 0.8–1.7 | N-Term, C-Term, Internal | Metal chelate | Yes or No (Denaturants) | Yes (Imidazole) or No (Low pH) | B, Y, I, M, P |
| | Polyarginine | 5–15 | 0.8–2.4 | C-Term | Cation-exchange resin | Yes | Yes (NaCl gradient at alkaline pH) | B, P |
| Antibody–epitope interactions | Hemagglutinin (HA) | 9 | 1.1 | N-Term, C-Term | IgG | Yes | No (Low pH) | Y, M |
| | T7 | 11 | 1.1 | N-Term | IgG | Yes | No (Low pH) | B, Y, M, P, I |
| | Synthetic protein A (Z domain) | 58 | 7 | N-Term | IgG | Yes | No (Low pH) | B, Y, I, M, P |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FLAG | 8 | 1.0 | N-Term, C-Term | mAb M1 and mAb M2 | Yes | Yes (EDTA) | B, Y, I, M, P |
| | c-myc | 11 | 1.2 | N-Term, C-Term | IgG | Yes | Yes (free c-myc epitope) | B, Y, I, M, P |
| Protein–protein interactions | Biotin | ~20–100 | ~2–11 | N-Term, C-Term | Modified avidin or streptavidin | Yes | Yes (20 mM Biotin) | B, I, M |
| | Calmodulin-binding peptide | 26 | 3 | N-Term, C-Term | Calmodulin | Yes (+$Ca^{2+}$) | Yes (+EGTA) | B |
| | Strep-tag II | 8 | 1.1 | N-Term, C-Term, Internal | Strep-tactin | Yes | Yes (desthiobiotin) | B, Y, I, M, P |
| | Streptavidin-binding peptide | 38 | 4 | C-Term | Streptavidin | Yes | Yes (biotin) | B |
| | Thioredoxin | 109 | 11.7 | N-Term, C-Term | Phenylarsine oxide-agarose | Yes | Yes ($\beta$-mercaptoethanol) | B |
| | His-patch thioredoxin | 109 | 11.7 | N-Term, C-Term | Metal chelate | Yes or no | Yes (Imidazole) or No (Low pH) | B |
| | S-tag | 15 | 1.8 | N-Term, C-Term | S-protein | Yes | Yes (2 M Sodium Thiocyanate) | B, I, M |

[a] B = bacteria; Y = yeast; I = insect; M = mammalian; P = plant.

subcellular localization of gene products, determination of protein topology, cellular trafficking studies, and the investigation of protein–protein interactions. They are also important in protein purification and immobilization.

Antibody-binding partners are covalently coupled to agarose chromatography resins or magnetic glass beads. Immobilized antibodies tend to be less stable than many other affinity-binding partners due to their need for native structure. Elution under mild native conditions can be achieved by competitive displacement with free epitope, by proteolysis, or by exposure of the $Ca^{2+}$ dependent FLAG-tag to EDTA.

## Protein–Protein Interaction Affinity Tags

Several high-affinity and high-specificity protein–protein interactions identified in biological systems have been adapted for use as affinity tags. *In vivo* biotinylation enzymatically couples biotin, a small molecule vitamin, to a protein acceptor domain. The resulting modified protein provides an interesting example of a natural affinity tag. A number of well-characterized protein sequences are enzymatically biotinylated *in vivo*. These natural peptides of approximately 100 residues have been used as fusion tags that direct *in vivo* biotinylation of a fusion protein in a site-specific manner. Additionally, shorter synthetic peptides (approximately 20 residues) known as biotin acceptor peptides (BAPs) have been developed for use as biotin affinity tags.

The high-affinity, specific interaction between biotin and either avidin or streptavidin ($K_D \sim 10^{-15}$ M) serves as the basis for immobilization. Biotin-tagged fusion proteins are typically captured by binding to monomeric avidin or streptavidin coupled to a chromatography matrix or other surface. Elution with free biotin requires harsh conditions that can be detrimental to the target protein, but proteolytic elution can be carried out under mild conditions. Alternatively, the biotin-tag fusion can be immobilized to modified forms of avidin or streptavidin that exhibit lower biotin affinity. Elution can then be conducted under nondenaturing conditions by competitive displacement with free biotin or alkaline pH.

Other examples of protein–protein affinity tags include the Strep-tag, the calmodulin-binding peptide (CalBP), and the S-tag. The Strep-tag is a nine amino acid peptide that was developed as an artificial ligand for streptavidin. Further refinements to these molecules yielded the eight amino acid peptide Strep-tag II and a mutant form of streptavidin called Strep-Tactin capable of moderate-affinity binding ($K_D \sim 1$ μM). Strep-tag fusion proteins are bound to streptavidin under physiological conditions and are efficiently displaced by free biotin or a biotin derivative. The mild conditions used for binding and elution enable Strep-fusions to be utilized in their native state. This small, stable, and nonperturbing tag is ideal for many applications in which a small tag is required, and for applications where detection of the fusion protein on a Western blot or by ELISA is desired. Moreover, since the Strep-tag is not itself a metalloprotein and its elution does not require metal chelators, it is well suited for metalloprotein applications.

The 26 amino acid calmodulin-binding peptide (CalBP) was derived from the C-terminus of skeletal muscle light-chain kinase. The CalBP binds to calmodulin with high affinity ($K_D \sim 1$ nM) in the presence of low $CaCl_2$ concentrations ($\geq 0.2$ mM). The binding partner, calmodulin, is commercially available linked to chromatography resins that are used to immobilize CalBP fusions. CalBP fusion proteins are eluted under mild conditions by a $Ca^{2+}$ chelating agent such as EGTA. CalBP fusions are primarily expressed in *E. coli* that lacks calmodulin family members and thus possesses no sequences evolved to bind to calmodulin. Eukaryotic cells are not recommended for expression of CalBP-tagged proteins due to their large number of endogenous proteins (approximately 30) that bind to calmodulin.

The S-tag protein fusion system consists of the S-peptide and its binding partner the S-protein, both derived from RNAase A. The S-tag binds to the S-protein with moderate affinity ($K_D \sim 100$ nM), resulting in a strong interaction that is influenced by pH, temperature, and ionic strength. A unique feature of the S-tag system is that ribonucleolytic activity is restored when the S-peptide is bound to the 103 amino acid S-protein. This enzymatic activity is exploited to measure the molar concentration of the S-tag fusion protein down to 20 fM in a highly sensitive and rapid assay. S-protein-based reagents have been developed to probe SDS-PAGE blots for S-tag fusions employing colorimetric or chemiluminescent detection of the target protein down to nanogram quantities.

## See Also the Following Articles

Affinity Chromatography • Two-Hybrid Protein–Protein Interactions

## Glossary

**epitope tag** Short polypeptide fused to a protein of interest so that it will be recognized as a high-affinity binding target by a specific, well-characterized antibody.

**fusion protein** Polypeptide made from a recombinant gene consisting of two or more gene fragments fused together.

**immobilized metal-affinity chromatography (IMAC)** A type of affinity chromatography based on the specific interaction between a metal chelate stationary phase and a metal-binding peptide fused to a protein of interest.

## Further Reading

Bornhorst, J., and Falke, J. (2000). Purification of proteins using polyhistidine affinity tags. *Methods Enzymol.* **326**, 245–254.

Jarvic, J., and Telmer, C. (1998). Epitope tagging. *Annu. Rev. Genet.* **32**, 601–618.

Nilsson, J., Ståhl, S., Lundeberg, J., Uhlén, M., and Per-Åke, N. (1997). Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein Expr. Purif.* **11**, 1–16.

Sheibani, N. (1999). Prokaryotic gene fusion expression systems and their use in structural and functional studies of proteins. *Prep. Biochem. Biotechnol.* **29**, 77–90.

Terpe, K. (2003). Overview of tag protein fusions: From molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol.* **60**, 523–533.

## Biography

Joseph J. Falke is Professor of Chemistry and Biochemistry, and Chair of the Molecular Biophysics Program, at the University of Colorado, Boulder. His research interests are in the area of signal transduction, in particular the mechanisms of cellular chemotaxis pathways in bacterial and eukaryotic systems. He holds a Ph.D. in chemistry from the California Institute of Technology and carried out his postdoctoral research at the University of California, Berkeley. His laboratory has made fundamental discoveries regarding the molecular mechanisms of receptors and signaling proteins involved in chemical sensing.

John A. Corbin is a Postdoctoral Fellow in the Falke Laboratory at the University of Colorado, Boulder. He holds a Ph.D. in biology from the University of California, Santa Cruz. His research interests are in the general areas of protein structure, function, and mechanism.

# A-Kinase Anchoring Proteins

**Lorene K. Langeberg and John D. Scott**
*Howard Hughes Medical Institute, Vollum Institute, Oregon Health and Sciences University, Portland, Oregon, USA*

**The precise transmission of information from a plasma membrane receptor to the downstream target inside the cell is essential for the control of dynamic cellular functions. It has been proposed that the coordination of signaling pathways inside cells is achieved, in part, by the localization of signaling enzymes such as kinases and phosphatases near their intended protein substrates. The organization of these enzymes into signaling scaffolds facilitates the phosphorylation state of specific proteins at appropriate time and place.**

## Protein Phosphorylation

Protein phosphorylation is a predominant form of covalent modification of proteins inside cells. This bidirectional process, catalysed by protein kinases and reversed by phosphoprotein phosphatases, provides a flexible means of influencing the proteins that control cellular metabolism, transcription, division, and movement. The utility of this regulatory mechanism is underscored by evidence that ~30% of intracellular proteins are phosphoproteins.

### Protein Kinase A

One well-studied "protein phosphorylation pathway" is regulated by the second messenger cAMP. When extracellular messengers bind to heptahelical receptors on the surface of the cell and recruit heterotrimeric G proteins to activate adenylyl cyclases on the inner face of the plasma membrane cAMP synthesis is triggered. This newly synthesized "second messenger" then diffuses to its sites of action. Although cAMP can modulate a few classes of signaling molecules, the predominant intracellular receptors are cAMP dependent protein kinases (PKA). The PKA holoenzyme is composed of two catalytic (C) subunits that are held in an inactive state by association with a regulatory (R) subunit dimer. The C subunits are expressed from three genes; C$\alpha$, C$\beta$, and C$\gamma$, whereas the R subunits are transcribed from four genes; RI$\alpha$, RI$\beta$, RII$\alpha$, and RII$\beta$. The type I PKA (composed of RI dimers) is predominantly cytoplasmic and is most highly expressed in the immune system, whereas type II PKA (composed of RII dimers) associates with cellular structures and organelles and is abundant in the heart and brain.

### Phosphorylation Specificity

One unresolved issue in cAMP signaling is the question of how this commonly used pathway is able to selectively regulate so many different cellular processes. For that reason, the mechanism by which PKA discriminates among its substrates is a topic of considerable interest. One hypothesis proposes that specific pools of the kinase are compartmentalized within the cell and are activated in close proximity to particular substrates. This can only occur if there is a means to both (1) selectively control the level of subcellular pools of cAMP and (2) maintain PKA in these environments. It has been proposed that a balance between adenylyl cyclase and phosphodiesterase activities leads to the establishment of intracellular gradients of cAMP. An equally important component of this model requires "scaffolding" proteins called "A-kinase anchoring proteins" that keep the kinase in close proximity to its substrates. This article will discuss the compartmentalization of the protein kinase A and other enzymes through their association with A-kinase anchoring proteins (AKAPs).

## The PKA Anchoring Hypothesis

The first AKAPs that were identified remained tightly associated with the type II R subunits during purification from tissues and were therefore designated "RII-binding proteins". Over 30 AKAPs have now been identified and are recognized as a family of diverse proteins that are classified on the basis of their interaction with the PKA inside cells. It has also become apparent that most AKAPs share some other common properties (Figure 1). These include a common R subunit binding sequence (Figure 1A), localization regions that target the PKA/AKAP complex to precise intracellular environments (Figure 1B) and binding sites for other enzymes to form

 © 2004, Elsevier Inc. All Rights Reserved. 

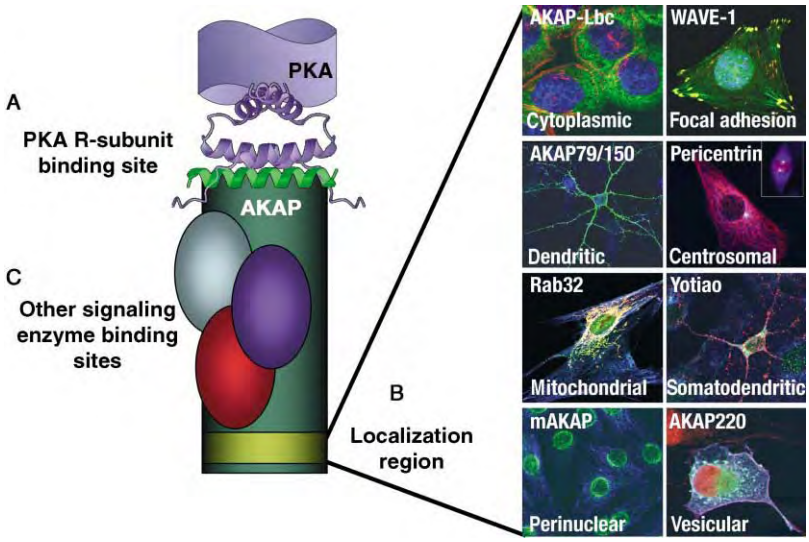



**FIGURE 1** Properties of A-kinase anchoring proteins. (A) A common identifying characteristic of AKAPs is a protein–protein interaction site that binds to protein kinase A. The amphipathic helix of the AKAP (green) tightly associates with the regulatory subunit amino terminal dimer of PKA (purple). (B) The localization region of the AKAP (yellow) is responsible for the precise subcellular targeting of the scaffold. Here we show the subcellular staining pattern for eight different AKAPs (in green, or yellow and white when colocalized with other stained proteins) found at distinct locations within the cell. (C) Multiple signaling enzymes associate with an AKAP via protein–protein interactions, nucleating a unique signaling complex for efficient signal transmission.

signaling complexes (Figure 1C). Each property is discussed here.

## Protein Kinase A Binding

### RII Subunit Binding Sequences

Most AKAPs contain a short sequence that forms a binding site for the R subunit dimmer and was first recognized in the human thyroid anchoring protein, AKAP-Lbc. The region likely forms an amphipathic helix that slots into a binding pocket formed by the amino terminal regions of RII. This view is supported by evidence that a 24-amino acid peptide encompassing this region, called Ht31, binds RII with low nanomolar affinity. Cellular delivery of this peptide antagonizes PKA anchoring and has become a standard means to delineate a role for AKAPs in the coordination of cAMP-responsive events. Recently, a new and improved anchoring inhibitor peptide has been developed from a comprehensive analysis of 10 AKAP sequences. This 17-amino acid peptide, called "AKAP-is", selectively binds RII with subnanomolar affinity, efficiently disrupts PKA anchoring inside cells, and functions to block cAMP signaling to glutamate receptor ion channels in cells.

### RI Subunit Binding

Although most AKAPs associate with the type II PKA, it is now clear that some anchoring proteins also target the type I kinase. Yeast two-hybrid screening and affinity purification techniques have identified anchoring proteins that can interact with either RI or RII (designated dual function AKAPs) and, in a few instances, RI selective AKAPs have been reported. Apparently, RI anchoring also proceeds through an amphipathic helix although other determinants may contribute to the compartmentalization of the type I PKA holoenzyme. Recently, a single nucleotide polymorphism (SNP) that causes a valine to isoleucine mutation in the anchoring helix of D-AKAP-2 has been shown to increase RI-binding affinity threefold. Although the functional ramifications of this valine to isoleucine change are unclear it is more prevalent in the aging population. Detailed structural analyses will be necessary to define the differences between type I and type II PKA anchoring.

## Anchoring Protein Targeting Regions

While protein–protein interactions are responsible for the precise orientation of the kinase toward its substrates, it appears that protein–lipid interactions target the AKAP–PKA complex to the correct intracellular membranes and organelles. In the brain for example, repeat sequences that bind negatively charged phospholipids tether an AKAP called AKAP79/150 to the inner surface of synaptic membranes. In addition, protein–protein interactions with SAP97, an adapter protein that binds to the cytoplasmic tail of the AMPA

receptor ion channel, places the AKAP79/150-PKA complex in the vicinity of substrates. This elaborate molecular bridging facilitates the PKA phosphorylation of serine 845 in the cytoplasmic tail of GluR1. Serine 845 is an important regulatory site on the channel that is modified during chemically induced long term potentiation (LTP). The AKAP79/150 complex also includes the phosphatase PP2B which functions to dephosphorylate serine 845 leading to attenuation of GluR1 channels. In fact, peptide disruption of the PP2B-AKAP79/150 interaction prevents efficient dephosphorylation of the channel and suggests that targeting of the phosphatase with its substrate is necessary for modulation of channel activity. In a similar manner myristoylation and palmitoylation signals facilitate the protein–lipid tethering of another AKAP, AKAP15/18 in close proximity to PKA substrates such as calcium channels and sodium channels. AKAP15/18 may also be cross-linked to the $\alpha_1$-subunit of the L type $Ca^{2+}$ channel via a modified leucine zipper motif.

There are instances where multiple AKAPs mediate PKA targeting to the same organelle. Three anchoring proteins (D-AKAP-1/AKAP149, D-AKAP-2, and Rab32) anchor PKA at mitochondria, two AKAPs (AKAP350-450/CG-NAP and pericentrin) tether the kinase to centrosomes whereas Ezrin, WAVE-1 and AKAP-Lbc tether PKA to distinct areas of the actin cytoskeleton. One explanation for these apparent redundancies may be the need to always maintain an anchored pool of PKA at certain sites. Alternatively, each compartment specific AKAP may direct the kinase to different microenvironments where specific substrates reside. Thus, compartmentalization of PKA is likely to be a more finely organized process than was initially appreciated.

## Scaffolding Complexes

### Multiple Enzyme Pathways

Perhaps the most important feature of AKAPs is their ability to simultaneously interact with several signaling proteins (Figure 1C). By localizing PKA with enzymes such as protein phosphatases, phosphodiesterases, G proteins, and other protein kinases, AKAPs provide focal points for the integration and processing of distinct intracellular signals. The notion was first proposed for the AKAP79/150 family, which maintains PKA, protein kinase C (PKC) and the phosphatase PP2B at the synaptic membrane. Subsequently, it has been shown that most, if not all, AKAPs nucleate signaling protein networks. For example, Yotiao, AKAP220, and AKAP149, tether protein phosphatase 1 to oppose the action of anchored kinases. This creates an environment where protein phosphorylation is only favored when kinase activity is sufficiently stimulated to overcome these basal dephosphorylation events. One variation on this theme occurs when signal termination enzymes that act upstream of protein kinases are recruited to AKAP signaling complexes. For example, AKAP450 and mAKAP co-localize a cAMP-metabolizing enzyme, the phosphodiesterase PDE4D3, with PKA. This creates a local environment where PDE activity reduces cAMP levels in the vicinity of the kinase. Presumably, these signaling complexes not only contribute to the formation of intracellular gradients of cAMP but also confer temporal control on PKA activation by generating pulses of kinase activity.

### Parallel Signaling Pathways

Other AKAP complexes are known to participate in the parallel processing of distinct intracellular signals. In the brain WAVE-1 is a scaffolding protein that principally functions to relay signals from the plasma membrane via the small molecular weight GTPase Rac to the Arp2/3 complex, a group of seven related proteins that nucleate actin polymerization and branching to facilitate remodeling events in the cytoskeleton. However, WAVE-1 also anchors PKA and binds to the Abelson tyrosine kinase (Abl). Proteomic approaches have identified additional binding partners that are positive and negative regulators of WAVE-1 function and substrates for either kinase. Thus WAVE-1 is capable of recruiting different combinations of signaling enzymes to the neuronal cytoskeleton for control of distinct protein phosphorylation and actin-remodeling events. This multifaceted role is reflected in the complex phenotype of WAVE-1 knockout mice which exhibit abnormalities in their brain morphology as well as behavioral defects that may be linked to altered signaling in the cerebellum or hippocampus.

### Alternatively Spliced Scaffolds

The AKAP350/450/CG-NAP/Yotiao family arise from alternative splicing of a single gene on chromosome 7q21. At least four anchoring proteins that are targeted to three distinct subcellular locations are transcribed from this gene. Initially a cDNA was isolated that encodes a 350 kDa protein believed to be a high molecular weight AKAP previously identified in centrosomal fractions. Around the same time variants encoding AKAP450 and CG-NAP were identified. The latter protein was named CG-NAP on the basis of its detection in centrosomal and Golgi fractions. Detailed analysis of CG-NAP has identified additional binding partners that include protein phosphatase 2A, the Rho-dependent-protein kinase PKN and the protein kinase C epsilon isoform. Functional studies propose that enzymes in this signaling complex may participate in membrane trafficking, microtubule nucleation and/or cell cycle progression.

Yotiao, the shortest splice variant in this family is targeted to synaptic sites where it anchors PKA and the type 1 protein phosphatase PP1 to regulate the phosphorylation state of NMDA receptor ion channels. Tonic PP1 activity negatively regulates NMDA receptors by favoring the dephosphorylated state. However, upon PKA activation the PP1 activity is overcome and the channel is phosphorylated resulting in increased NMDA receptor currents. More recently a requirement for yotiao targeting of PKA and PP1 to GABA(A) receptors at inhibitory synapses has been demonstrated in the dopaminergic regulation of cognitive processes. Thus, AKAP350/450, CG-NAP and yotiao organize PKA and a plethora of signaling enzymes in a variety of subcellular locations. Transcriptional regulation is undoubtedly a critical determinant for location and composition of each signaling complex maintained by these AKAP gene products.

## Understanding the Function of Scaffolds

An emerging area of investigation is the genetic manipulation of AKAPs. Although several anchoring proteins have been identified in genetically tractable organisms including *C. elegans*, *D. melanogaster*, and *D. rerio* (zebrafish) the most significant advances have come from the characterization of genetically modified mice. Genetic disruption of the MAP2 gene causes a redistribution of the PKA holoenzyme in neurons that limits certain cAMP responsive phosphorylation events and causes reduction in dendrite length. In a similar manner disruption of the WAVE-1 gene has apparent effects on brain morphology and effects complex neuronal behaviors including coordination, balance, learning, and memory. These observations complement previous evidence that ablation of PKA subunit genes alters hippocampal-based forms of learning and memory.

Traditionally, these anchoring molecules were thought to exclusively control cAMP responsive events. However AKAP-mediated compartmentalization of other signaling enzymes may be an equally important function. Recent reports have implicated AKAP350/CG-NAP, AKAP220 and WAVE-1 networks in the control of Rho kinase signaling, glycogen synthase kinase 3 action and Rac mediated actin remodeling respectively. As the detailed dissection of these AKAP signaling complexes progresses it seems probable that their role in the coordination of both cAMP dependent and independent signaling events will become more evident.

## See Also the Following Articles

Cyclic Nucleotide-Dependent Protein Kinases • Cyclic Nucleotide Phosphodiesterases • Glycogen Synthase Kinase-3 • Protein Kinase C Family

## Glossary

**cytoskeleton** The complex network of actin microtubules and microfilaments in the cytoplasm that provide structure to the cytoplasm of the cell and plays an important role in cell movement and maintaining the characteristic shape of the cells.

**phosphorylation/dephosphorylation** The activation of an enzyme by the addition of a phosphate group to the enzyme or the inactivation of an enzyme by the removal of a phosphate group from the enzyme.

**protein kinase** The enzyme that catalyzes the transfer of phosphate from ATP to the hydroxyl side chains of a protein causing changes in the function of the protein.

**protein phosphatase** An enzyme that catalyzes the removal of phosphate from a phosphorylated protein, thereby causing changes in the function of the protein.

**receptor** Proteins located either on the cell surface or within the cytoplasm that bind ligand, initiating signal transduction and cellular activity.

**scaffold** A protein that serves as a platform to bring together a unique assortment of signaling enzymes for the efficient transmission of intracellular messages.

## Further Reading

Bauman, A. L., and Scott, J. D. (2002). Kinase- and phosphatase-anchoring proteins: Harnessing the dynamic duo. *Nat. Cell Biol.* **4**(8), E203–E206.

Cohen, P. (2000). The regulation of protein function by multisite phosphorylation – A 25 year update. *Trends Biochem. Sci.* **25**(12), 596–601.

Colledge, M., and Scott, J. D. (1999). AKAPs: From structure to function. *Trends Cell Biol.* **9**(6), 216–221.

Hunter, T. (1995). Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225–236. (January 27).

Pawson, T., and Nash, P. (2000). Protein–protein interactions define specificity in signal transduction. *Genes Dev.* **14**(9), 1027–1047.

Pawson, T., and Scott, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075–2080.

Scott, J. D., and Pawson, T. (2000). Cell communication: The inside story. *Sci. Am.* **282**(6), 72–79.

Skalhegg, B. S., and Tasken, K. (2000). Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA. *Front Biosci.* **5**, D678–D693.

## Biography

Lorene K. Langeberg earned her B.A. from the University of Colorado at Boulder followed by work with Jon Lindstrom at The Salk Institute. She moved to John Scott's laboratory at the Vollum Institute in 1992 and is currently a Research Specialist with the Howard Hughes Medical Institute.

John D. Scott received his Ph.D. in Biochemistry from the University of Aberdeen prior to postdoctoral work with Edwin Krebs at the University of Washington. In 1990 he joined the faculty at the Vollum Institute at Oregon Health and Science University where he is now a Senior Scientist and an Investigator with the Howard Hughes Medical Institute.

# Allosteric Regulation

**Barry S. Cooperman**
*University of Pennsylvania, Philadelphia, Pennsylvania, USA*

**Allosteric regulation refers to the process for modulating the activity of a protein by the binding of a ligand, called an effector, to a site topographically distinct from the site of the protein, called the "active site," in which the activity characterizing the protein is carried out, whether catalytic (in the case of enzymes) or binding (in the case of receptors) in nature. The word allosteric, Greek for "other site," was coined to emphasize this distinctness. The modulation of protein activity is accomplished by the reversible alteration of the protein conformation that accompanies effector binding. Effectors that increase activity are called activators, while those that decrease activity are called inhibitors. For the purposes of this article we will use the term substrate to indicate a ligand bound to the active site of either an enzyme or a receptor that undergoes the characteristic activity of the protein.**

## Allosterism and Cooperativity

Allosteric regulation has been found to be extensive in proteins, particularly in enzymes at key branch points of metabolism and in receptors that must be sensitive to small variation in signals. Although a monomeric protein having one subunit can display allosteric regulation, the great majority of proteins regulated in this manner have multiple subunits, with changes in activity arising from changes in subunit–subunit contacts. A characteristic feature of these regulatory proteins is the occurrence of cooperative interactions for both the substrate and the regulatory ligand. This property renders their function dependent upon threshold concentrations of ligand.

### Cooperative Binding of $O_2$ to Hemoglobin

Cooperativity may be defined as any process in which an initial event affects subsequent similar events. It was initially identified with the sigmoid plot for the binding of four molecules of $O_2$ to hemoglobin (Hb) (Figure 1), a pseudotetrameric protein that gives red blood cells their color. A similar cooperativity of substrate binding occurs in many allosteric enzymes, leading to sigmoid plots of enzyme activity versus substrate concentration. Hb has the subunit composition $\alpha_2\beta_2$, in which the $\alpha$- and $\beta$-subunits are nearly identical to one another, with each containing a heme group to which $O_2$ binds. The sigmoid plot was explained by the concept that the first molecule of $O_2$ bound makes it easier for subsequent molecules to bind, and so is an example of positive cooperativity. In fact, if the Hb-binding curve is fitted to four successive binding constants, the affinity for the fourth $O_2$ bound is calculated to be 100–1000 times as high as the first $O_2$ bound. In contrast, $O_2$ binding to myoglobin (Mb), a monomeric protein found in vertebrate muscle with no possibility for site-site interaction, follows a rectangular hyperbola plot.

An essential feature of the positive cooperativity, shown in Figure 1 for Hb, is that it sharpens the responsiveness of a system to a change in substrate (or effector) concentration. Thus, to go from 10% to 90%, $O_2$ saturation of Mb requires an 81-fold change in $O_2$ concentration, whereas the corresponding change for $O_2$ saturation of Hb requires only a fourfold change. Such responsiveness is obviously desirable in a protein whose activity must be highly regulated.

### The Hill *n*, a Measure of Cooperativity

The Hill plot linearizes saturation data over the major portion of the saturation curve (typically 10–90% saturation) yielding a slope, called the Hill $n$ and denoted $n_H$, which provides a convenient empirical measure of cooperativity. If we define the saturation function $Y_S$ as the fraction of all binding sites containing a bound ligand (i.e., $0 \leq Y_S \leq 1$) then the Hill plot is obtained by plotting $\log(Y_S/(1 - Y_S))$ versus $\log [S]$ (when enzyme activity is measured, $\log(v/(V_{max} - v))$ versus $\log [S]$ is plotted instead). The value of $n_H$ is one for a noncooperative saturation function (e.g., $O_2$ binding to Mb). Positive cooperativity is defined by an $n_H > 1$, with an upper limit of $n$, the number of identical subunits, for a cooperative saturation function. For a tetramer, an $n_H$ equal to its upper limit of 4 would correspond to the situation where there were only two protein species in solution, one with no ligands bound

 © 2004, Elsevier Inc. All Rights Reserved. 

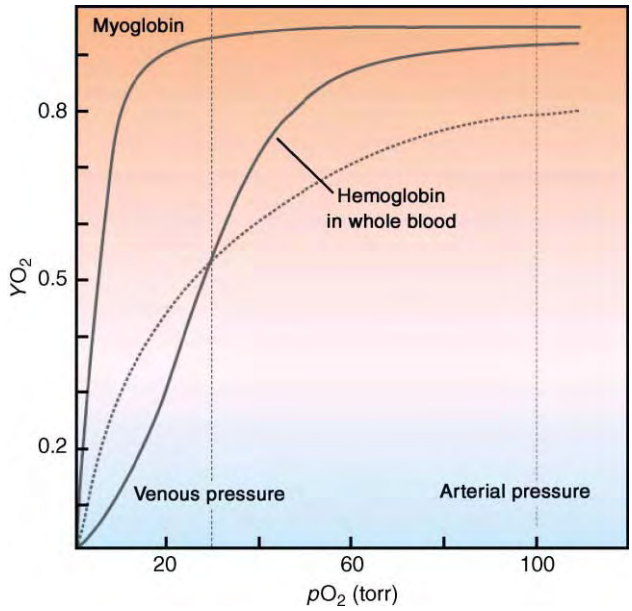



**FIGURE 1** The $O_2$ binding curves for Hb (cooperative) and Mb (noncooperative). The dashed line represents a noncooperative curve for $O_2$ binding having the same value as Hb for the $O_2$ pressure ($pO_2$) required for half-saturation ($YO_2 = 0.5$). Adapted from Voet, D., Voet J. G., and Pratt, C. W. (2002) *Fundamentals of Biochemistry*. Wiley, New York.

and with ligands bound to all four sites. For Hb, a strongly cooperative tetramer, the $n_H$ value is near 3 under physiological conditions. Some allosteric proteins have $n_H < 1$, i.e., have negative cooperativity. In contrast to positive cooperativity, negative cooperativity gives a less sensitive response over a broad range of stimulus and might be important, for example, in the response of growth or general metabolism to changes in hormone concentration.

One caveat to the use of $n_H$ values evaluated from enzyme activity studies is that hysteresis or ligand-induced slow transitions that have the same timescale as the catalytic cycle can give rise to apparent cooperativity, even for monomeric enzymes. In addition, $n_H$ values of $< 1$, whether determined by binding or enzyme activity studies, can arise from sample heterogeneity, rather than from true negative cooperativity.

## Two-State Models to Explain Cooperativity in Allosteric Proteins

Two models were proposed in the 1960s to explain not only the cooperative binding of $O_2$ to Hb, and of substrate binding to allosteric proteins in general, but also the effects of allosteric effector molecules on substrate binding. Each of the models posits that the identical subunits within the protein can exist in two states, $T$ and $R$, with the $R$ state having higher activity for substrate. Here some definitions are in order before proceeding further. $O_2$ binding to hemoglobin is an example of a "positive homotropic effect," since the initial binding of $O_2$ increases the affinity for subsequent $O_2$ molecules. On the other hand, addition of the allosteric inhibitor 2,3-diphosphoglycerate reduces $O_2$ affinity for Hb, providing an example of a "negative heterotropic effect." In a similar fashion, an allosteric activator which increases substrate affinity gives rise to a positive heterotropic effect, whereas a negative homotropic effect is seen when initial binding of substrate decreases the affinity for subsequent substrate molecules.

The first model, proposed by Monod, Wyman, and Changeux in 1965, and known as the MWC (or symmetry) model hypothesizes that: (1) allosteric regulatory proteins, in general, are oligomers made up of a finite number of identical subunits that occupy equivalent positions and, as a consequence, possess at least one axis of rotational symmetry; (2) the allosteric oligomers can exist in two freely interconvertible and discrete conformational states ($T$ or $R$) that differ in the energy of their intersubunit interactions, but in which molecular symmetry is conserved, so that all subunits are either in the $T$ state or the $R$ state; (3) in the absence of ligand, the pre-existing conformational equilibrium is characterized by an allosteric constant $L = (T_n)/(R_n)$, where $n$ is equal to the number of identical subunits; and (4) ligand affinities for the active and allosteric sites carried by the oligomers may differ between the two states, allowing ligand binding to preferentially stabilize the state for which it exhibits a higher affinity. Such modulation of the conformational equilibrium by ligand binding suffices to generate positive homotropic effects and both positive and negative heterotropic effects.

The second model, proposed by Koshland, Nemethy, and Filmer in 1966, and known as the KNF (or sequential) model, hypothesizes that in the absence of ligand the protein exists in a single state, that ligand binding induces a conformational change only in the subunit to which it binds, and that cooperative interactions arise through the influence of such conformational changes on intersubunit interaction. The KNF model embodies the notion of "induced fit," whereby the binding of substrate to an active site causes conformational changes of active site residues that are necessary for the protein's function. Since conformational change only occurs on ligand binding, partially saturated protein contains a mixture of $R$ and $T$ states, so that the symmetry of the oligomeric protein is not preserved during the binding process.

Both the MWC and KNF models are limiting cases of the more general scheme of a two-state model, as shown for the case of substrate binding to a tetrameric protein in Figure 2. In the MWC model only species corresponding to the left-most ($T_nS_i, i = 0 - n$) and right-most

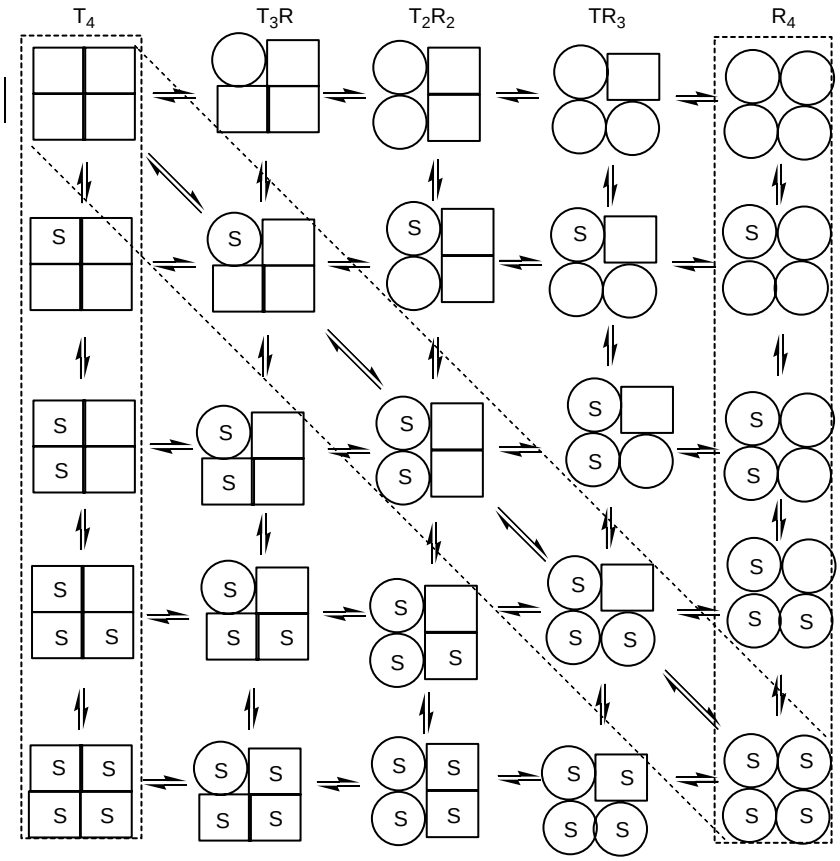



**FIGURE 2** The general two-state model for substrate binding to an allosteric tetrameric protein. The columns on the left and right show the species included in the MWC model. The diagonal from upper left to lower right shows the species included in the KNF model.

($R_nS_i, i = 0 - n$) columns are considered, because it is posited that hybrid states containing both R- and T-subunits are inherently unstable and do not accumulate. In contrast, in the KNF model only species lying along the diagonal connecting $T_n$ to $R_nS_n$ (i.e., $T_{n-i}R_iS_i, i = 0 - n$) are considered, because of the requirement that conformational change only results from substrate binding.

Either limiting model generates the sigmoidal saturation curve for substrate binding to an allosteric protein using just three parameters. For the MWC model these are $L$, the allosteric constant, and $K_T$ and $K_R$, the dissociation constants for S binding to the $T$ and $R$ states, respectively. In the KNF model the three parameters are $K_SK_t$ (as a product), $K_{RT}$, and $K_{RR}$, where $K_S$ is the binding constant for substrate to the $R$ state, $K_t$ is the equilibrium constant for changing a single, isolated, subunit from the $T$ to the $R$ conformation (= [$R$]/[$T$]) and $K_{RT}$ and $K_{RR}$ measure the relative strength of associations of $R-T$ and $R-R$ intersubunit contacts, respectively, versus the strength of association of $T-T$ intersubunit contact. In the two models, allosteric effectors exert their heterotropic effects by altering the apparent conformational equilibrium constant ($L'$ or $K_t'$) in each model, with inhibitors, which bind $T$ state preferentially, increasing $L'$ or decreasing $K_t'$ and activators, which bind $R$ state preferentially, decreasing $L'$ or increasing $K_t'$.

Because of their relative simplicities, both the MWC and KNF models provide useful frameworks for the analysis of allosteric proteins, although, in detail, each enzyme studied may show deviations. In some cases one model may be preferred over the other. Thus, the MWC model does not account for negative homotropic effects ($n_H < 1$), whereas such effects can arise in the KNF model when $K_{RT}$ is substantially greater than either $K_{TT}$ or $K_{RR}$. On the other hand, the MWC predicts that the state function $R$, which measures the fraction of all subunits in the $R$ state, will, in general, increase more rapidly as a function of substrate concentration than the saturation function $Y_S$, since the binding of one substrate molecule can shift all the subunits in $T_n$ to the $R_n$ conformation. In contrast, the KNF model demands that $R$ is always equal to $Y_S$. Experimental examples have been found for both negative homotropic cooperativity and for $R \neq Y_S$. Detailed examination of structural or energetic changes on partial ligand binding have been used to differentiate between the two models.

### Extensions and Modifications of Two-State Models

When ligand binding induces a change in the oligomeric state of the protein, cooperativity can result as a direct consequence of ligand binding preferentially to either the associated or dissociated form. If, for example, substrate binding favors a higher state of oligomerization, then higher enzyme concentrations will enhance activity but depress cooperativity, and, at constant enzyme concentration, allosteric ligands that favor association will be activators while those that favor dissociation will be inhibitors. The equations to account for this type of behavior are similar in form to those generated by either the MWC or KNF models, but with the addition of terms that are dependent on protein concentration.

Thus far, we have considered allosteric proteins in which the substrate and effector molecules have different affinities for the *R* and *T* states. These are the so-called *K* (binding constant) systems. In pure *K* allosteric enzymes, the *T* and *R* states have identical $V_{max}$ values. However, allosteric enzymes can also be regulated by *V* (velocity) systems. In pure *V* allosteric enzymes, the *R* and *T* states have identical affinity for substrate but different $V_{max}$ values. In both *K* and *V* enzymes an effector functions by binding preferentially to the *R* or *T* state. For *K* systems, such binding results in altered affinity and cooperativity of substrate binding. In *V* sytems, effector binding results in altered $V_{max}$ values, and there is no cooperativity in substrate binding.

## Structures of Allosteric Proteins

High-resolution structures have been determined for a number of allosteric enzymes in both the *R* and *T* states, permitting some generalizations to be made and allowing critical consideration of the MWC and KNF models. One common finding is that ligand-binding sites, both active site and regulatory, are located at subunit interfaces. Such placement provides an exquisite means of communicating cooperative and/or allosteric effects between subunits in an oligomeric enzyme, since sites at the interface are likely to respond to the changes in subunit interactions that are critical for allosteric regulation. For similar reasons, a second common location for ligand binding is at the interface of two domains within same subunit. Placement of the active site at either interface has as a consequence that even modest conformational changes can cause significant changes in the size and shape of the active site, thus altering the protein's activity. Allosteric binding sites also tend to be located in low-stability regions of the protein. The binding of an effector stabilizes both the region itself and specific contacts the region makes with adjacent regions, requiring the movements of polypeptide backbone and side chains, and, in particular, the alteration of salt bridges and hydrogen bonds at subunit interfaces. These movements provide a mechanism for transmission of signals to the active site and to other allosteric sites over distances of tens of angstroms. Other common features are: (1) small but critical movements at the active site; (2) the rotation of subunits with respect to one another around a cyclic axis; (3) only two modes of subunit:subunit docking, consistent with the MWC model of concerted transition; and (4) a more highly constrained and extensive subunit interface in the less active *T*-state than in the *R*-state.

## Examples of Allosteric Proteins Other than Hemoglobin

### Aspartate Transcarbamoylase (ATCase)

ATCase catalyzes a key step of pyrimidine biosynthesis, the condensation of carbamoyl phosphate with aspartate to form N-carbamoylaspartate. The *Escherichia coli* enzyme has been extensively studied. CTP is an allosteric inhibitor representing a classic case of feedback inhibition whereby the end product of a biosynthetic pathway inhibits an enzyme catalyzing a reaction at the beginning of the pathway. ATP is an allosteric activator, and together CTP and ATP act on ATCase to coordinate the rates of purine and pyrimidine nucleotide biosynthesis. The enzyme has the subunit composition $c_6r_6$, where *c* and *r* are catalytic and regulatory subunits, respectively. The *c* subunits are arranged as two $c_3$ s, which are complexed with three $r_2$ s. In the absence of *r* subunits, the *c* subunits are catalytically active, and are unaffected by ATP or CTP, which bind only to the *r* subunit. Crystal structures have shown that the $c_6r_6$ holoenzyme exists in two conformations, with CTP preferentially binding to the inactive *T*-state and ATP to the active *R*-state. Interestingly, ATP and CTP bind competitively to the same allosteric site in the *r* subunit. CTP binding induces a contraction in $r_2$, which in turn, via interactions at the $r-c$ interface, results in the $c_3$ s moving together by 0.5Å. This movement causes a perturbation of the active site and a loss in catalytic activity. In contrast, ATP binding results in the $c_3$ s moving apart by 0.4Å.

### Ribonucleotide Reductase (RR)

RRs form a family of allosterically regulated enzymes that catalyze the conversion of ribonucleotides to deoxyribonucleotides and are essential for *de novo* DNA biosynthesis. Their allosteric regulation is designed to match the flux of the four deoxynucleotides produced with the base composition of the organism's DNA.

Class Ia RRs are the most widespread in nature. They accept the four common nucleoside diphosphates (NDPs) as substrates, with enzymatic activity dependent upon the formation of a heterocomplex between subunit R1, which contains the active site and three allosteric sites (the s-, a-, and h-sites), and subunit R2, which contains a stable tyrosyl-free radical that is necessary for NDP reduction at the active site. The substrate specificity of RR is determined by the allosteric ligand occupying the s-site. ATP and dATP stimulate the reduction of CDP and UDP, dTTP stimulates the reduction of GDP and dGTP stimulates the reduction of ADP. The s-site is located at the interface between R1 monomers, such that effector binding drives formation of $R1_2$. The regulation of total enzyme activity is controlled by ATP and dATP, chiefly at the level of changes in oligomerization state. Both bind to the a-site, located at the interface between two $R1_2$ s, driving formation of $R1_4$, which exists in two conformations, $R1_{4a}$ and $R1_{4b}$, with the latter predominating at equilibrium, while only ATP binds to the h-site, which drives formation of $R1_6$. Only the $R2_2$ complexes of $R1_2$, $R1_{4a}$, and $R1_6$ are enzymatically active. dATP is a universal inhibitor of RR activity due to its induction of $R1_{4b}$ formation whereas ATP is a universal activator because it induces $R1_6$ formation. Class II RRs, which are only found in bacteria, are considerably simpler, comprising only a monomeric protein containing an active site, an s-site and a $B_{12}$ cofactor. Interestingly, the active site and s-site have the same relative orientations as in Class Ia, with the s-site located at an interdomain interface.

## GroEL

*E. coli* GroEL is an example of a chaperonin, which mediates protein folding in an MgATP-dependent manner. GroEL consists of 14 identical subunits that form two heptameric rings. Cooperativity in ATP binding and hydrolysis by chaperonins reflects the switching of rings between protein-binding and protein-release states and is important for regulation of their reaction cycle. GroEL displays two levels of allostery: one within each ring and the second between the two rings. In the first level of allostery, each heptameric ring is in equilibrium between $T$ and $R$ states that interconvert in a concerted manner, in accordance with the MWC model. In the absence of ligands, GroEL is predominantly in the $T_7T_7$ state. In the presence of low concentrations of ATP ($<100\ \mu$M), the equilibrium is shifted toward the $T_7R_7$ state, displaying positive cooperativity in ATP binding and hydrolysis. A further shift in the equilibrium from the $T_7R_7$ state to the $R_7R_7$ state ($L2 = [RR]/[TR]$) takes place only at higher ATP concentrations because of inter-ring negative cooperativity. This second level of allostery is better described by the KNF model.

## Cell-Surface Receptors Involved in Signal Transduction

Membrane receptors for transmitters, peptides, and pharmacological agents are central to signal transduction. They selectively recognize chemical effectors (neuronal or hormonal) and allosterically transduce binding recognition into biological action though the activation of ligand-gated ion channels (LGICs) and/or G-protein-coupled receptors (GPCRs). Various features of membrane receptors for neurotransmitters are well accommodated by the MWC model. These receptor proteins are typically heterooligomers and exhibit transmembrane polarity. In general, the regulatory site to which the neurotransmitter binds is exposed to the synaptic side of the membrane while the biologically active site is either a transmembrane ion channel, a G protein-binding site, or a kinase-catalytic site facing the cytoplasm. Interactions between the two classes of sites are mediated by a transmembrane allosteric transition. Signal transduction or activation is mediated by a concerted cooperative transition between a silent resting state and an active state. Agonists stabilize the active state and competitive antagonists the silent state, and partial agonists may bind nonexclusively to both. These receptors can also undergo a cascade of slower, discrete allosteric transitions, which include refractory regulatory states that result in the desensitization or potentiation of the physiological response.

## See Also the Following Articles

Chaperonins • Ligand-Operated Membrane Channels: Calcium (Glutamate) • Ligand-Operated Membrane Channels: GABA • Pyrimidine Biosynthesis

## Glossary

**allosteric effector** A molecule binding to a protein site other than the active site, which can either increase or decrease the activity of the protein.

**heterotropic cooperativity** The effect of an allosteric effector on protein activity toward a substrate.

**homotropic cooperativity** The effect of initial substrate binding on subsequent substrate binding.

**KNF and MWC models** Thermodynamic models that account for the cooperativity displayed by allosteric enzymes.

## Further Reading

Changeux, J.-P., and Edelstein, S. J. (1998). Allosteric receptors after 30 years. *Neuron* **21**, 959–980.

Cooperman, B. S., and Kashlan, O. B. (2003). A comprehensive model for the allosteric regulation of class in ribonucleotide reductases. *Adv. Enzyme Regulation* **43**, 167–182.

Eaton, W. A. E., Henry, E. R., Hofrichter, J., and Mozzarelli, A. (1999). Is cooperative oxygen binding by hemoglobin really understood? *Nature Struct. Biol.* **6**, 351–358.

Fersht, A. (1999). *Structure and Mechanism in Protein Science*. WH Freeman, New York.

Horovitz, A. (2001). Review: Allostery in chaperonins. *Journal of Structural Biology* **135**, 104–114.

Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966). *Biochemistry* **5**, 365–385.

Luque, I., and Freire, E. (2000). Structural stability of binding sites: Consequences for binding affinity and allosteric effects. *PROTEINS: Structure, Function, and Genetics* **41**(suppl 4), 63–71.

Monod, J., Wyman, J., and Changeux, J. P. (1965). *J. Mol. Biol.* **12**, 88–118.

Neet, K. E. (1995). Cooperativity in enzyme function: Equilibrium and kinetic aspects. *Methods of Enzymology* **249**, 519–567.

Voet, D., and Voet, J. G. (2004). *Biochemistry*, 3rd edition, Vol. 1, Wiley, New York.

Voet, D., Voet, J. G., and Pratt, C. W. (2002). *Fundamentals of Biochemistry*. Wiley, New York.

## Biography

Dr. Barry S. Cooperman is a Professor of Chemistry at the University of Pennsylvania, where he previously also served as Vice Provost for Research. His broad interests are in the fields of biological and bioorganic chemistry, with a special focus on the mechanisms of action of ribosomes, ribonucleotide reductase, and serpins, and has published numerous articles in these fields. He holds a Ph.D. from Harvard University and was a postdoctoral fellow with Jacques Monod at the Institut Pasteur.

# Alternative Splicing: Regulation of Fibroblast Growth Factor Receptor (FGFR)

**Mariano A. Garcia-Blanco**
*Duke University Medical Center, Durham, North Carolina, USA*

**Most stable biological programs and many dynamic biological responses rely on the quantitative and qualitative regulation of gene expression. Such regulation can be accomplished by rearranging the gene, controlling transcription of the primary RNAs, regulating the processing of these primary transcripts into mature RNA species, modulating the stability and distribution of these mature RNAs, and finally, in some cases, manipulating the synthesis and stability of the protein products templated by messenger RNAs. Among these steps in the information flow of gene expression, this article focuses on the regulation of RNA processing and, in particular, alternative splicing. Alternative splicing is the process by which a single species of primary transcript undergoes differential removal of introns to yield different mature RNAs. Alternative splicing, which affects gene products from the majority of protein-coding genes, is likely one of the major engines of proteome diversity.**

## Premessenger RNA Splicing

The initial RNA products of transcription by RNA polymerase II (RNAPII), primary transcripts, are large molecules (averaging 30,000 nucleotides) that are divided into exons and introns. Exons, which usually average 300 nucleotides in length, are retained in mature RNAs, whereas introns, which average over 3000 nucleotides, are removed from the primary transcripts by RNA splicing. RNA splicing occurs in the nucleus and appears to be coupled to the synthesis of primary transcripts by RNAPII. RNA splicing is catalyzed by the spliceosome, an RNA-based macromolecular enzyme that recognizes and defines the exons and introns and precisely removes the latter and rejoins the exons (Figure 1). The definition of exons and introns is governed by conserved cis-acting elements: the 5′ splice site (also known as the donor site) demarcating the 5′-end of an intron and the 3′ splice site (also known as the acceptor site), a tripartite element marking the 3′-end of an intron. It should be noted that although the definition focuses on introns, all internal exons (e.g., exon M in Figure 1D) are defined by a 3′- splice site upstream and a 5′- splice site downstream.

## Alternative Splicing

There are four types of alternative splicing events in which splice site choice solely determines the sequence of mature RNAs (Figures 1A–D). In rare cases, an intron is removed or retained to give two very different RNAs (A). The use of two or more alternative 5′ splice sites (B) or 3′ splice sites (C) can lead to RNA isoforms. Finally, inclusion or skipping of one or more exons is a common form of alternative splicing (D). In addition, alternative splicing of transcripts initiated at different transcription start sites leads to mature RNAs with different first exons (E). The 3′ terminal exons can also vary by coupling alternative splicing with alternative polyadenylation (F). This entry describes a slightly more complex form of the decision to skip or include an exon (D), which is observed among transcripts encoding the fibroblast growth factor receptor-2 (FGFR2).

## Alternative Splicing of FGF-R2 Transcripts

The extracellular region of FGF-R2 has three immunoglobulin-like (Ig) domains and the third Ig domain determines ligand-binding specificity. This third domain comes in two forms, which differ only in the sequence of the C-terminal half of the domain. One form, predominant in cells of epithelial origin, is encoded by sequences within exons 7 and 8 (IIIb), while the other IgIII domain isoform, which is expressed in fibroblasts, is encoded by sequences within exons 7 and 9 (IIIc) (Figure 2). Alternative splicing of the same FGF-R2 primary transcript, via the mutually exclusive use of exons 8 (IIIb) or 9 (IIIc), leads to the production of FGF-R2

 © 2004, Elsevier Inc. All Rights Reserved. 

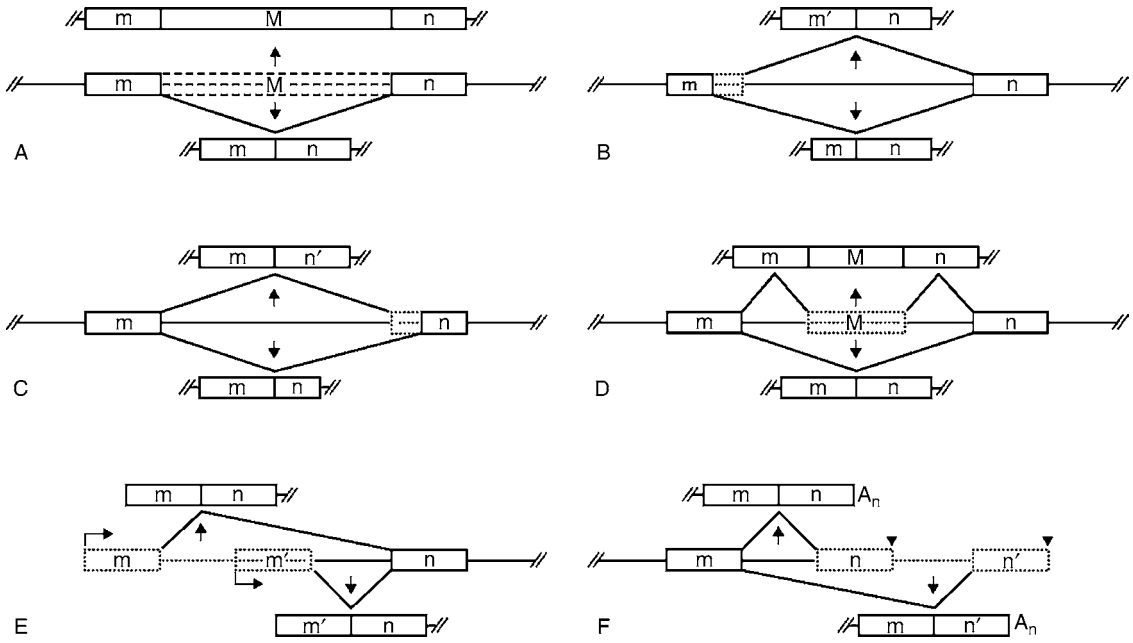



**FIGURE 1** Forms of alternative splicing. (A–D) Cases in which transcript diversity is provided solely by alternative splicing. (E) A case in which alternative transcription-initiation sites coupled with alternative splicing leads to diversity of products. (F) A case in which alternative splicing and alternative 3′-end formation lead to diverse transcripts.

(IIIb) or FGF-R2 (IIIc). The importance of this alternative splicing is suggested by the following: (1) FGF-R2 (IIIb) and FGF-R2 (IIIc) bind different FGFs; (2) the expression of two isoforms is tightly regulated in different cell types; (3) the targeted disruption of exon 8 (IIIb) or exon 9 (IIIc) in transgenic animals leads to nonoverlapping defects in organogenesis and development, which are not compatible with viability; and finally (4) several genetic disorders in humans have been mapped to mutations in IgIII of FGF-R2. The regulation of the mutually exclusive use of exons IIIb or IIIc is mediated by cis-acting elements within the primary transcript and trans-acting factors, which must differ in different cell types.

## Regulatory Elements and Factors

The cis-acting regulatory elements that control alternative splicing can be divided generally into four categories: exonic splicing enhancers, exonic splicing silencers, intronic activators of splicing, and intronic splicing silencers. These cis-elements mediate their function by interacting, directly or indirectly, with trans-acting activators or repressors of splicing.

### Exonic Splicing Enhancers

Exonic splicing enhancers (ESEs) are cis-acting elements that activate the definition of an otherwise weak

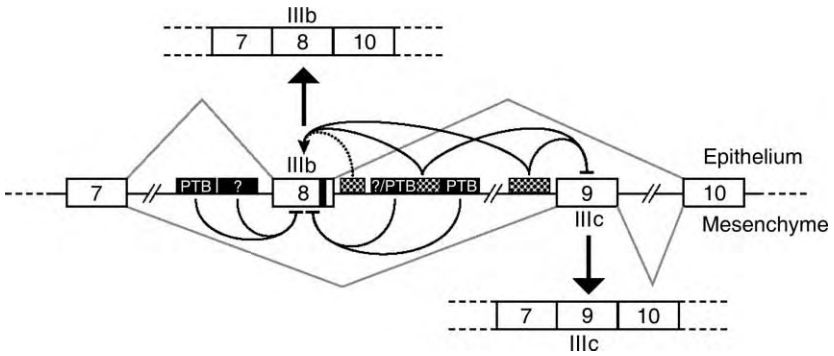



**FIGURE 2** Multiple layers of combinatorial interactions result in tissue-specific alternative spliced transcripts: the example of fibroblast growth factor receptor-2. A schematic of exons 7–10 and introns 7–9 of FGF-R2 transcripts is shown indicating that in mesenchymal cells a layer of negative regulation, which includes weak splices bordering exon 8, an exonic splicing silencer (black bar in exon 8), and flanking intronic splicing silencers (black rectangles) silence exon 8 and lead to the choice of exon 9. In epithelial cells, however, a second layer of regulation combines activation of exon 8 by intronic activators of splicing (stippled rectangles) with repression of exon 9 (stippled rectangles) to ensure the use of exon 8.

exon, promoting its inclusion into mature transcripts. Several families of ESEs have been recognized, and the most common are characterized by purine-rich sequences (consensus) and mediate their action via members of the SR protein family (e.g., SC35). Although ESEs have been implicated in regulated splicing, they appear to have a role in defining many constitutive exons. The FGF-R2 IgIII alternative splicing unit does not have obvious ESEs (Figure 2).

## Exonic Splicing Silencers

Exon definition can be repressed by exonic splicing silencers (ESSs). The best-characterized ESSs bind hnRNP A1, a protein originally described as an abundant heterogeneous nuclear RNA-binding protein. HnRNP A1 binding at ESSs can suppress inclusion of a weak exon, as has been noted for exon IIIb of FGF-R2 transcripts (Figure 2). HnRNP A1 and SR proteins can play counterbalancing roles in exon definition and their relative levels may determine tissue-specific alternative splicing outcomes.

## Intronic Activators of Splicing

Intronic activators of splicing (IASs), which can provide constitutive or regulated enhancements of exon definition, can selectively activate weak exons. Exon IIIb definition requires IAS1, which is a long U-rich element found immediately downstream of the exon. IAS1 binds the Tia-1 (huNam8p) protein that provides ancillary activation of exon IIIb. This activation critically requires two other IASs, IAS2 and IAS3, which mediate epithelial cell-specific inclusion of the exon. Portions of IAS2 and IAS3 directly interact, forcing a double-stranded RNA stem that is required for activation (Figure 2). IAS2 and IAS3 are part of more complex elements that also mediate repression of exon IIIc in epithelial cells and are thus termed intronic splicing activators and repressors of splicing (ISARs) (Figure 2). The coordinated cell-type activation of exon IIIb and repression of exon IIIc results in the epithelial pattern of FGF-R2 splicing.

## Intronic Splicing Silencers

Intronic splicing silencers (ISSs) inhibit exon definition by directly occluding cis-elements within canonical splice sites or by creating zones of silence around exons. Many times, ISSs are found flanking exons subject to silencing; this is the case for exon IIIb. Two bipartite silencers, upstream and downstream of exon IIIb, are required for repression of this exon in fibroblasts. Both ISSs mediate their action via the polypyrimidine tract binding protein (PTB), another member of the hnRNP protein family, and other unidentified factors (Figure 2). The actions of the ISSs dominate in fibroblasts; however, in epithelial cells IASs antagonize the silencers. Silencing of introns, possibly in combination with a lack of ESEs, can be important to prevent inappropriate inclusion of pseudo exons, which have canonical splice sites with proper spacing but are not used as exons. These pseudo exons can serve as silent reservoirs of genetic information.

## Layers of Regulation

The alternative splicing of FGF-R2 exons IIIb and IIIc provide examples of ESSs, IAS, and ISS, and in addition reveal several general features of regulated alternative splicing units. First, regulation is mediated by the superimposition of several layers of control, starting with weakening of the canonical splice sites bordering regulated exons, followed by global silencing mediated

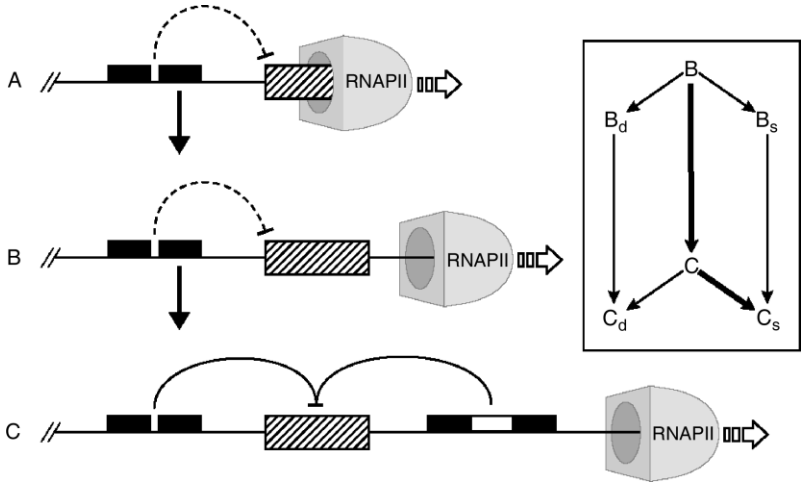



**FIGURE 3** A dynamic view of exon silencing. In many exons, for example, for exon 8 (IIIb) of FGF-R2 transcripts, flanking ISSs are required for silencing. (B) During the synthesis of the transcript there is a time when the exon is fully accessible to the splicing apparatus but either is not defined or is defined reversibly. (C) Synthesis of the downstream ISS leads to full repression. The inset proposes the view that the rate of synthesis (B → C) is faster than the rate of exon definition (B → $B_d$).

by non-cell-type-specific ESS and ISS, and finally capped by cell-type-specific ISAR elements. Second, the layers of regulation involve antagonistic interactions between activators and repressors of exon definition. Third, each individual layer is determined by a combination of cis-elements and factors. Fourth, a general principle that is far from being understood mechanistically, regulation must be coupled with transcription of RNAPII.

## A Dynamic View of Alternative Splicing

Most schematics of alternative splicing (including Figure 2) present a static splicing substrate. Although useful, this static view must give way to a more realistic representation. A dynamic representation of the silencing of exon IIIb is presented in Figure 3. This schematic focuses on the potential to define the exon, which can only proceed once the exon is synthesized and accessible to the splicing machinery (Figure 3B). Exon definition ($B \rightarrow B_d$) must be slow relative to the rate of transcript synthesis ($B \rightarrow C$). Upon the appearance of the downstream ISS full silencing can ensue (Figure 3C).

### See Also the Following Articles

Fibroblast Growth Factor Receptors and Cancer-Associated Perturbations • Pre-tRNA and Pre-rRNA Processing in Bacteria • RNA Polymerase II and Basal Transcription Factors in Eukaryotes • RNA Polymerase II Structure in Eukaryotes • RNA Polymerase Reaction in Bacteria • RNA Polymerase Structure, Bacterial

### Glossary

**alternative splicing** The process by which a single species of primary transcript undergoes differential removal of introns to yield different mature RNAs. Alternative splicing, which affects gene products from the majority of protein-coding genes, is probably one of the major engines of proteome diversity.

**exon** A block of sequence within the primary transcript that is retained in the mature transcript after splicing. Internal exons average 145 nucleotides in length among protein-coding transcripts.

**intron** A block of sequence, which averages ~3000 nucleotides in length but can be larger than 100,000 nucleotides, that is removed during splicing.

**RNA splicing** One of the processes by which primary transcripts (RNAs) are converted into mature transcripts. During RNA splicing, introns are removed and exons are precisely ligated together.

### Further Reading

Berget, S. M. (1995). Exon recognition in vertebrate splicing. *J. Biol. Chem.* **270**, 2411–2414.

Black, D. L. (2000). Protein diversity from alternative splicing: A challenge for bioinformatics and post-genome biology. *Cell* **103**, 367–370.

Fairbrother, W. G., Yeh, R. F., Sharp, P. A., and Burge, C. B. (2002). Predictive identification of exonic splicing enhancers in human genes. *Science* **297**, 1007–1013.

Garcia-Blanco, M. A., Lindsey, L. A., and Ghosh, S. (2001). The phosphoryl transfer reactions in pre-messenger RNA splicing. In *RNA* (D. Söll, S. Nishimura and P. Moore, eds.), pp. 109–123. Pergamon Press, San Diego, CA.

Goldstrohm, A. C., Greenleaf, A., and Garcia-Blanco, M. A. (2001). Co-transcriptional splicing of pre-messenger RNAs: Considerations for the mechanism of alternative splicing.*Gene* **277**, 31–47.

Maniatis, T., and Tasic, B. (2002). Alternative pre-mRNA splicing and proteome expansion in metazoans. *Nature* **418**, 236–243.

### Biography

Dr. Mariano A. Garcia-Blanco is Professor of Molecular Genetics and Microbiology, Professor of Medicine at Duke University Medical Center, and Co-director of the Duke Center for RNA Biology. His principal research interest is in the field of gene expression, specifically the interactions that connect transcription with pre-mRNA processing and the regulation of alternative splicing. He holds M.D. and Ph.D. from Yale University.

# Alternative Splicing: Regulation of Sex Determination in *Drosophila melanogaster*

**Jill K. M. Penn, Patricia Graham and Paul Schedl**
*Princeton University, Princeton, New Jersey, USA*

**Alternative splicing is a regulatory process that produces multiple mRNA transcripts from pre-mRNAs transcribed from a single gene. The net effect of alternative splicing is that diverse proteins can be manufactured from a limited amount of DNA. Often a cell contains distinct ratios of alternatively spliced mRNAs and, consequently, distinct ratios of protein isoforms. The ratio of isoforms can vary among different cell types and different stages of development. Conversely, alternative splicing can contribute to cell identity by producing tissue- or stage-specific mRNAs rather than distinct ratios. One example of this exists in *Drosophila melanogaster*, in which alternative splicing is responsible for sex-specific protein expression that functions to maintain sexual identity. Intriguingly, *Drosophila* sexual development is governed by alternative splicing at multiple levels of the regulatory hierarchy.**

## The Sex Determination Hierarchy in *Drosophila melanogaster*

### Sexual Choice, Memory, and Differentiation

The X chromosome/autosome ratio (X/A ratio) determines the choice of sexual identity early in embryogenesis by setting the activity state of the master regulatory switch gene *Sex-lethal* (*Sxl*). An X/A ratio of 1.0 specifies female identity by turning *Sxl* on, whereas an X/A ratio of 0.5 specifies male identity by keeping *Sxl* off (Figure 1). Once *Sxl* is activated in 2X/2A animals, it functions to maintain the female determined state through a positive autoregulatory feedback loop in which Sxl protein promotes its own expression and to orchestrate female development by directing the expression of several regulatory cascades in the female mode. In 1X/2A animals, male identity is remembered by the absence of Sxl protein, while male development is specified by the expression of these same regulatory cascades in the default mode. Although the initial choice of sexual identity is controlled at the level of transcription, many of the key regulatory steps for both memory and differentiation depend on sex-specific alternative splicing.

### Details of the Hierarchy

Although *Sxl* is on in females and off in males, the gene is transcribed in both sexes from a promoter, *Sxl-Pm*, which is active from the cellular blastoderm stage onwards. The critical difference between the sexes is that all *Sxl* mRNAs in males have an additional exon, exon 3, which is spliced out of the *Sxl* mRNAs in females (Figure 2A). This male-specific exon has multiple in-frame stop codons that prematurely truncate the *Sxl* open reading frame that begins at an AUG codon in exon 2. Consequently, male mRNAs do not encode functional proteins. In contrast, female *Sxl* mRNAs encode proteins that have two RNA recognition motif (RRM)-type RNA-binding domains. These proteins positively autoregulate their own synthesis by directing the splicing machinery to join exon 2 to exon 4, skipping the male-specific exon 3 (Figure 2A). Because female splicing never occurs in the absence of Sxl protein, the X/A ratio must activate the *Sxl* autoregulatory feedback loop in female embryos by a mechanism that bypasses this requirement. This is accomplished by turning on a special promoter, *Sxl-Pe*, in precellular blastoderm 2X/2A, but not 1X/2A embryos. The Sxl proteins encoded by the *Sxl-Pe* mRNAs set the autoregulatory feedback loop in motion in 2X/2A embryos by directing the female-specific splicing of the first *Sxl-Pm* transcripts. Because *Sxl-Pe* is not activated in 1X/2A embryos, the *Sxl-Pm* transcripts are spliced in the default male pattern.

The regulatory target for *Sxl* in the somatic sexual differentiation pathway is *transformer* (*tra*). The second exon of the *tra* pre-mRNA has two 3′ splice sites, the upstream default splice site and the downstream female-specific site (Figure 2B). Sxl protein turns *tra* on in

 © 2004, Elsevier Inc. All Rights Reserved. 

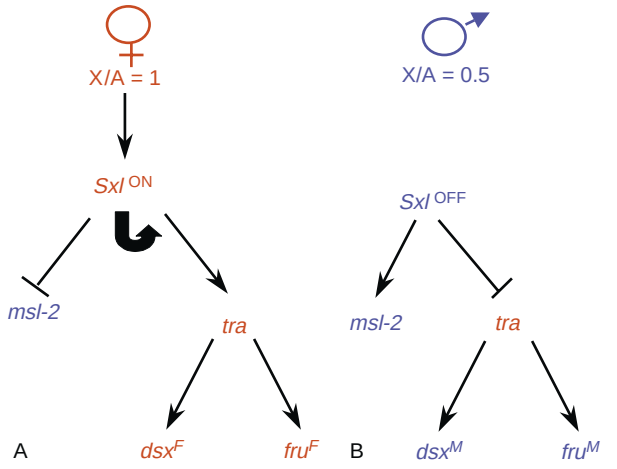



**FIGURE 1** Sex determination hierarchy in *Drosophila melanogaster*. (A) Female regulatory cascade showing that *Sex-lethal* is the master regulator of sex determination because it receives the initial signal that chooses sexual identity, maintains sexual identity by a positive autoregulatory feedback loop, and regulates sexual differentiation through downstream target genes. (B) Male regulatory cascade showing the opposite events leading to male development.

females by promoting splicing to the downstream 3′ splice site of exon 2. This produces an mRNA that has an open reading frame that encodes a functional Tra protein. When Sxl protein is absent, as in males, the default 3′ splice site is used. Because there is an in-frame stop codon just downstream of the default 3′ splice site, the male *tra* mRNA does not encode a functional protein and *tra* is off.

Like *Sxl*, *tra* promotes female differentiation by regulating splicing. The two known targets of Tra, *double-sex (dsx)* and *fruitless (fru)*, encode transcription factors. In the case of *dsx*, *tra* controls the production of sex-specific isoforms that have a common N-terminal DNA-binding domain, but different C-terminal activation domains. In females Tra promotes the joining of the 5′ splice site of exon 3 to the 3′ splice site of exon 4 (Figure 2D). In males, where Tra is absent, splicing is to the default 3′ splice site of exon 5. Although Tra regulates *dsx* pre-mRNA splicing by controlling the use of alternative 3′ splice sites, it regulates *fru* pre-mRNA splicing by controlling the use of alternative 5′ splice sites. As illustrated in Figure 2E, a default upstream 5′ splice site in exon 2 is used in males, whereas in females, in which Tra is present, the splicing machinery skips this 5′ splice site and uses instead a 5′ splice spite located some 1500 bp further downstream. Although both the male and female mRNAs are predicted to encode functional protein, the female-specific mRNA does not appear to be translated.

In addition to controlling somatic sexual differentiation, *Sxl* regulates dosage compensation. To make up for the difference in dose of X-linked gene products in the two sexes, transcription from the X chromosome is hyperactivated in males by the male-specific lethal (MSL) dosage-compensation system. *Sxl* turns off the dosage compensation system in females by negatively regulating one of its components, *msl-2*. Interestingly, Sxl regulates *msl-2* by blocking the alternative splicing of an intron in the 5′-UTR and by inhibiting the translation of the message (Figure 2C).

## Alternative Splicing Regulation by *Sxl*

### *tra*: A Simple Blockage Model?

The regulation of *tra* splicing by *Sxl* is much simpler than autoregulation in that it only involves a choice between alternative 3′ splice sites (Figure 2B), and it will be considered first. In principle, Sxl could promote the use of the downstream female-specific 3′ splice site in the *tra* pre-mRNA by preventing the splicing machinery from using the upstream default 3′ splice site. Alternatively, it could activate the downstream 3′ splice site. Sosnowski *et al*. tested these models by making deletion constructs that were missing one of the two alternative 3′ splice sites of exon 2. Deletion of the default 3′ splice site led to use of the downstream female-specific 3′ splice site not only in females, but also in males in which Sxl protein is absent. This finding is inconsistent with a simple activation model because in this model Sxl protein would be required to use the downstream site even when the upstream site is missing. Deletion of the downstream 3′ splice site did not interfere with splicing in males, but led to an increased amount of unspliced mRNA in females. This result is consistent with a simple blocking model because when an alternative 3′ splice site is not present, Sxl protein still inhibits the use of the default 3′ splice site, leading to an increase in unspliced mRNA.

Biochemical studies suggest that Sxl blocks the use of the default 3′ splice site by competing with the generic splicing factor U2AF$^{50}$. U2AF$^{50}$ normally binds to polypyrimidine tracts where it facilitates spliceosome assembly by interacting with the U2 small nuclear ribonucleoparticle (snRNP). Although U2AF$^{50}$ can bind to the polypyrimidine tracts of both the default 3′ and female-specific splice site, it binds with much higher affinity to the default polypyrimidine tract. The default polypyrimidine tract is unusual in that it contains two polyuridine (poly U) runs. Poly U is the recognition sequence for the Sxl protein and mutations in the *tra* poly U runs that eliminate Sxl protein binding *in vitro* prevent *Sxl* from regulating splicing *in vivo*. Thus, the classic model of *tra* splicing is that the Sxl protein outcompetes U2AF$^{50}$ for binding to the polypyrimidine tract of the default 3′ splice site, forcing U2AF$^{50}$ to bind to and activate the weaker, downstream 3′ splice site.

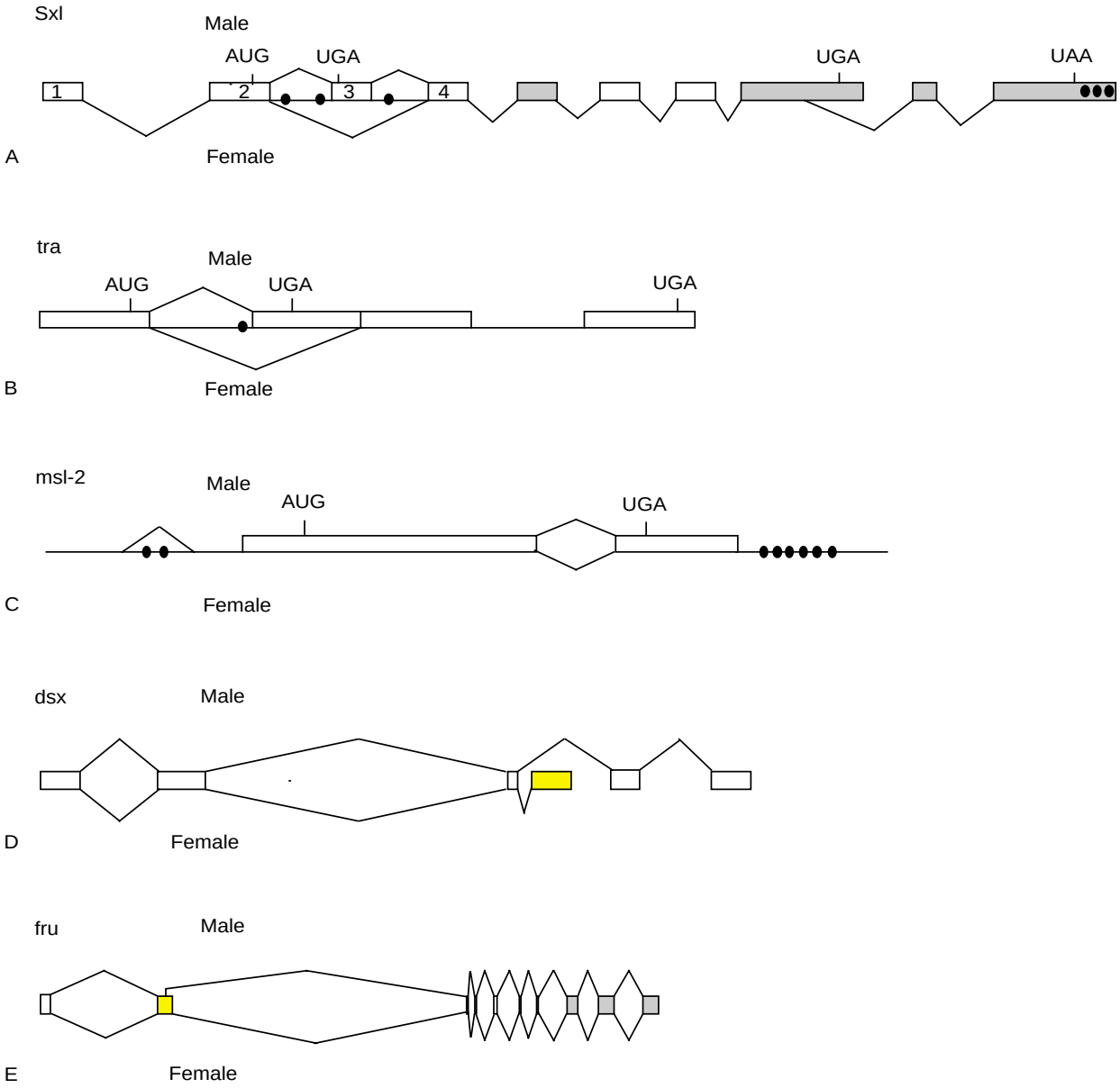



**FIGURE 2** Sex-specific alternative splicing. The sex-specific splicing patterns for (A) Sxl, (B) tra, (C) msl-2, (D) dsx, and (E) fru. The male splicing pattern is shown above the gene, and the female pattern is below. Ovals indicate Sxl binding sites. Yellow exons in dsx and fru contain RE and PRE binding sites. Sxl and fru have multiple promoters, and some regions of these genes undergo non-sex-specific splicing (gray). The establishment promoter of Sxl, controlled by the X/A ratio, is not sex-specifically spliced. For fru, only transcripts from promoters that are alternatively spliced in a sex-specific manner are indicated.

Consistent with this model, when the N terminus of Sxl protein is replaced with the U2AF activation domain, the hybrid Sxl protein binds to poly U sites and activates splicing to the default 3′ splice site.

Because a protein consisting of only the two Sxl RRM domains can bind to the default polypyrimidine tract with higher affinity than U2AF[50], this competition model predicts that only the RNA-binding domain of the Sxl protein should be necessary to direct female-specific splicing of *tra*. Indeed, this is the case in *in vitro* splicing reactions. However, a Sxl protein containing the RNA-binding domains, but lacking the first 40 amino acids is unable to regulate *tra* splicing *in vivo* in transgenic animals. One explanation for this discrepancy is that the binding of Sxl to the poly U runs in the default polypyrimidine tract must be stabilized *in vivo* by protein–protein interactions involving the N-terminal domain. However, this reasoning would not explain why a protein consisting of the N-terminal 100 amino acids of Sxl fused to $\beta$-galactosidase (N-$\beta$gal) is able to weakly promote female-specific splicing of *tra* when expressed in male flies.

This N-βgal fusion protein does not have the two Sxl RNA binding domains and consequently cannot directly compete with U2AF$^{50}$ for binding to the polypyrimidine tract of the default 3′ splice site. Taken together, these findings suggest that in addition to competing with U2AF$^{50}$ for binding to the default polypyrimidine tract, Sxl may play a more active role in directing the splicing machinery to use the downstream female-specific 3′ splice site. As described later, Sxl is known to associate with a number of components of the splicing machinery *in vivo*, including U2AF. Potentially such interactions could provide a mechanism for directing assembly of a functional spliceosome on the weak downstream splice site.

## *Sxl* Autoregulation

Although autoregulation is also thought to involve some type of blockage mechanism, it must be more complicated than *tra* splicing because Sxl has to regulate the use of both the 3′ and 5′ splice sites of the male exon (Figure 2A). Like *tra*, the polypyrimidine tract at the 3′ splice site of the *Sxl* male exon has a poly U run that is recognized by Sxl protein. However, because mutations in this poly U run have no effect on the splicing of *Sxl* pre-mRNAs in either tissue culture cells or transgenic animals, one can rule out a mechanism in which Sxl directs female splicing by binding to the polypyrimidine tract of the male exon and directly occluding U2AF or other generic splicing factors.

In fact, splicing regulation does not seem to pivot on controlling the use of the male exon 3′ splice site. When the male exon 5′ splice site is deleted, placing the 3′ splice sites of the male exon and exon 4 in direct competition, the male exon 3′ splice site is skipped in *both* sexes and exon 2 is spliced exclusively to exon 4. A different result is obtained when the 5′ splice sites of exon 2 and the male exon are placed in competition by deleting the male exon 3′ splice site. In males, a new male exon is generated using a cryptic 3′ splice site in the second intron and the normal male exon 5′ splice site. This deletion mutant transcript is also appropriately regulated by Sxl in females where exon 2 is spliced directly to exon 4, skipping the cryptic male exon. Other findings also argue that controlling the use of the male exon 5′ splice site is likely to be more important than controlling the 3′ splice site.

One mechanism to make the 5′ splice site of the male exon preeminent is to perturb the functioning of the male exon 3′ splice site. The male exon is unusual in that it has two 3′ splice site AGs, located 18 nucleotides apart, that compete with one another and reduce the efficiency of splicing. Use of the male exon in a heterologous context can be improved by mutating the upstream AG so that it can no longer be used. In the context of *Sxl*, inactivating the upstream AG compromises the ability of Sxl to block the inclusion of the male exon, presumably because the male exon 3′ splice site now functions much more efficiently. Finally, Lallena *et al.* have recently shown that the downstream AG is recognized by U2AF, whereas the upstream AG is recognized by the splicing factor SPF45. As would be predicted, if SPF45 is important for splicing to the upstream AG, reducing the activity of SPF45 in tissue culture cells (with RNAi) substantially increases the use of the downstream 3′-AG. The consequent improvement in efficiency of splicing of exon 2 to the male exon 3′ splice site compromises the ability of Sxl to block the inclusion of the male exon.

How does Sxl regulate the male exon 3′ and 5′ splice sites? It turns out that there are multiple poly U runs that are critical for regulation, located some 200 bp from the male exon. The deletion of the upstream or downstream poly U runs greatly compromises, but does not completely abolish, regulation by Sxl, whereas regulation is eliminated when both are deleted. Because the critical poly U runs are located far from the regulated splice sites, it is thought Sxl exerts its effect on male exon splicing indirectly through interactions with other components of the splicing machinery. Consistent with this idea, a number of proteins have been found in complexes with Sxl, and mutations in the genes encoding these proteins show genetic interactions with *Sxl*. The best characterized of these is *sans-fille* (*snf*), which encodes the *Drosophila* homologue of the mammalian U1 and U2 snRNP proteins U1A and U2B′. Although recombinant Sxl and Snf interact directly with one another *in vitro*, the immunoprecipitable complexes between Snf and Sxl seen in nuclear extracts are RNase-sensitive and do not include the U1 and U2 snRNPs unless the extracts are cross-linked prior to immunoprecipitation. Cline *et al.* proposed a snRNP-independent function for Snf in regulating *Sxl* splicing because they found that increasing the levels of *snf*, but not other genes encoding U1 or U2 snRNP proteins, could promote *Sxl* autoregulation under conditions of limiting Sxl protein. However, recent genetic experiments by Nagengast *et al.* show that Sxl interacts with the Snf protein associated with U1 snRNPs. The same study has also shown that Sxl can form an RNA-independent complex with the U1 snRNP protein U170K, and genetic interactions indicate that this protein–protein interaction is important for autoregulation.

The association of Sxl with two U1 snRNP proteins suggests that Sxl may prevent splicing between the male exon and exon 4 by blocking the assembly of a functional U1 complex at the male exon 5′ splice site. However, this would not explain how Sxl prevents exon 2 from splicing to the male exon 3′ splice site(s). An understanding of the role of Sxl in this context is becoming clearer from studies done with U2AF.

In nuclear extracts, Sxl can form a complex with U2AF$^{50}$ and U2AF$^{38}$, and *Sxl* mutations interact genetically with U2AF$^{38}$. Naggengast *et al.* suggest that one mechanism Sxl might use to block splicing between exon 2 and the male exon would be through interactions with the U2AF heterodimer. Because U2AF recognizes the downstream AG in the male exon 3′ splice site, some other mechanism might be required to block the use of the upstream AG.

Genetic and molecular studies indicate that genes encoding two additional trans-acting factors, *fl(2)d* and *virilizer* (*vir*), are necessary for regulating the female-specific splicing of *Sxl* (and of *tra*), although how these proteins function is unclear. *fl(2)d* is homologous to *wilm*'s *tumor associated protein* (*wtap*) and encodes a protein present in human spliceosomes. In *Drosophila*, the Fl(2)d protein can form an RNA-independent complex with Sxl, and the two proteins are able to interact directly *in vitro*. Interestingly, Fl(2)d is able to form an RNA-independent complex with Snf, which would be consistent with Fl(2)d functioning with the U1 or U2 snRNP because Snf is a component of both. *vir*, on the other hand, is not homologous to any known gene involved in splicing, but Vir protein has been shown to be in a complex with Fl(2)d, suggesting that Vir might function in splicing regulation.

## Alternative Splicing Regulation by Tra

### Tra Protein

Tra protein is an arginine/serine domain containing splicing regulator (SR) protein. SR proteins generally function to activate splice sites, but there are examples of SR proteins that are able to block splice sites instead. In *Drosophila*, female-specific Tra protein functions with sex-nonspecific Tra-2 protein to activate splice sites in *dsx* and *fru* pre-mRNAs.

### Regulation of *dsx* and *fru*

In males, the default splicing machinery joins the 5′ splice site of *dsx* exon 3 to the 3′ splice site of exon 5. When Tra is present in females, it promotes splicing of exon 3 to exon 4 by activating the 3′ splice site of exon 4. Exon 4 contains six 13-nt repeat elements (RE) and one purine-rich element (PRE) (located between RE #5 and RE #6) that are necessary for efficient use of the female-specific splice site. When these elements are deleted, *dsx* is spliced in the male pattern irrespective of whether Tra protein is present. Conversely, when these elements are placed downstream of a heterologous 3′ splice site they can activate the splice site when Tra is present. *In vitro* experiments indicate that Tra and Tra-2, together with the SR protein RBP1, associate with the 13-nt REs, whereas the SR proteins dSRp30 and b52/dSRp55 function at the PRE. It is thought Tra and Tra-2 plus the various SR proteins form an enhancer complex in exon 4. Because Tra and Tra-2 interact with U2AF$^{38}$, this suggests a model in which the enhancer complexes assembled in exon 4 recruit U2AF$^{38}$ and subsequently U2AF$^{50}$ to the 3′ splice site.

This model is complicated by the fact that the *fru* transcript contains three similar Tra/Tra-2 binding sites within exon 2, which are necessary for activating a female-specific 5′ splice site. This is difficult to reconcile with the U2AF recruitment model because recruiting U2AF is not useful for activating a 5′ splice site. Moreover, when the six REs and the PRE within exon 4 of *dsx* are replaced with the Tra/Tra-2 binding sites found in the *fru* transcript, activation of the female-specific 3′ splice site still occurs. One explanation for this result is that recruitment of general splicing regulators by Tra, Tra-2, and other SR proteins might be context dependent. Tra, Tra-2, and specific SR proteins might be able to recruit different proteins to activate either a 3′ or a 5′ splice site. Which component of the splicing machinery is recruited may be determined by additional proteins specific to each transcript that bind to sites other than the REs and/or PREs. Alternatively, the U2AF recruitment model might be entirely incorrect and Tra and Tra-2 might use a similar mechanism to activate both 5′ and 3′ splice sites.

## Alternative Splicing and Sex Determination in Other Species

*Sex-lethal* is produced in a sex-specific manner within the genus *Drosophila*, but in every other dipteran examined outside of this genus, *Sex-lethal* is not expressed sex-specifically nor does it appear to have a role in sex determination. By contrast, some of the downstream genes seem to maintain their role in sex determination. In *Ceratitis capitata*, *tra* seems to act as the master regulator of sex determination. *Cctra* appears to receive the initial sex-determination signal and subsequently regulates not only the necessary downstream gene(s) in the hierarchy of sexual development, but also maintains its own expression via alternative splicing. Further down the hierarchy, the gene *dsx* is much more evolutionarily conserved. In both *Megaselia scalaris* and *C. capitata*, *dsx* is structurally conserved and sex-specifically alternatively spliced. Intriguingly, the *dsx* homologue in *Caenorhabditis elegans*, *male abnormal 3* (*mab-3*), also controls sexual cell fate, thus extending the conservation beyond dipteran insects.

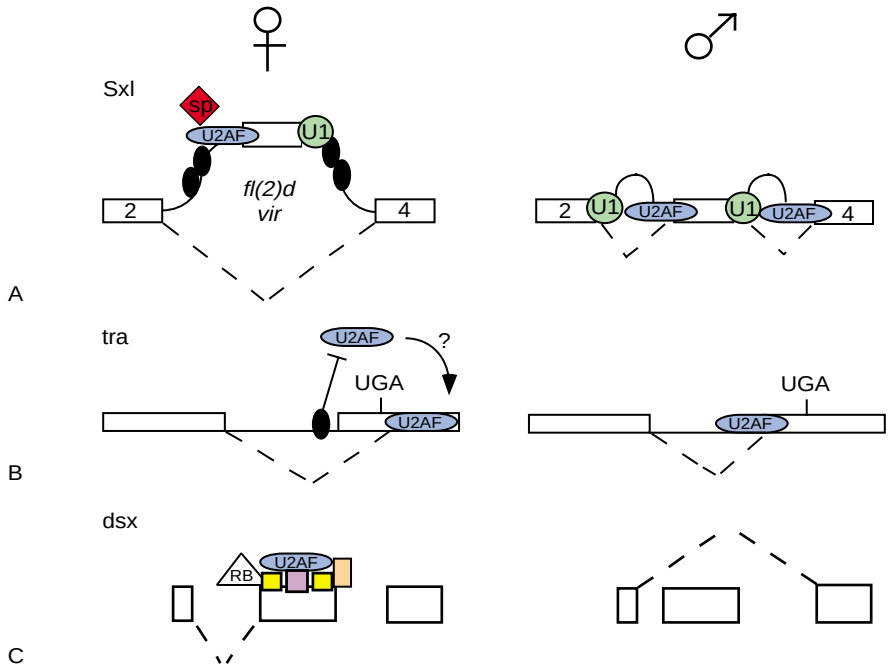



**FIGURE 3** Models for regulation of alternative splicing. Splicing patterns for (A) Sxl, (B) tra, and (C) dsx mRNAs in females (left column) and males (right column). Black ovals, Sxl; green circles, Ul snRNP (including snf and U170K); red diamond, spF45; yellow squares, tra/tra2; orange rectangle, dSRp30; purple square, b52/dSRp55; white triangle, RBP1. (A) To regulate splicing of its own mRNA, Sxl may bind to components of U2AF at the male 3′ splice site and components of the U1snRNP at the male 5′ splice site, thereby preventing these splice sites from being used. When no Sxl protein is present, the male exon splice sites are available. (B) To regulate tra splicing, Sxl competes with U2AF for binding to the poly(U) tract near the upstream 3′ splice site, forcing U2AF to use the weaker downstream site. Sxl may also activate the downstream 3′ splice site. When there is no Sxl, U2AF uses the stronger site. (C) Tra/tra-2 bound with the RE and PRE elements in exon 4 of doublesex may form a complex with other factors to activate the upstream 3′ splice site. When the complex is not present, the downstream site is used.

## Summary

*Drosophila* sex determination is a powerful and practical system in which to examine the mechanisms of alternative splicing. Studies done thus far indicate that within the *Drosophila* sex determination hierarchy are examples of several different mechanisms that control alternative splicing. *Sxl* autoregulation is controlled by blocking both the 3′ and 5′ splice sites of an exon, while female-specific *tra* expression is achieved by blocking the stronger 3′ splice site (Figure 3). Splice site activation is used for female-specific *dsx* and *fru* expression. However, a 3′ splice site is activated in *dsx*, whereas a 5′ splice site is activated in *fru*. Each of the various alternative splicing events is unique, but some of the proteins regulating these splicing reactions are shared. Sxl protein probably interacts with Fl(2)d and Vir to direct female-specific splicing of both *Sxl* and *tra*. Likewise, Tra and Tra-2 interact at similar consensus binding sites in the exons of *dsx* and *fru* pre-mRNAs. Further studies will hopefully elucidate which combinations of protein–protein interactions allow the same trans-acting factors to regulate two different alternative splicing events.

## See Also the Following Articles

Chromosome Organization and Structure, Overview • Spliceosome

## Glossary

**dosage compensation** A process that equalizes the amount of gene products when opposite sexes have different numbers of sex chromosomes.

**small nuclear ribonucleoparticle (snRNP)** A complex composed of proteins and snRNA (small nuclear RNA) that functions in the splicing reaction.

**spliceosome** A large complex composed of proteins and snRNPs that assembles on pre-mRNAs and catalyzes the splicing reaction.

**splicing regulator (SR) proteins** Proteins containing one or more arginine–serine (RS)-rich domains that often activate weak splice sites by protein–protein interactions.

## Further Reading

Cline, T. W., and Meyer, B. J. (1996). Vive la difference: Males vs females in flies vs worms. *Annu. Rev. Genet.* **30**, 637–702.

Cline, T. W. (1999). Functioning of the *Drosophila* integral U1/U2 protein Snf independent of U1 and U2 small nuclear ribonucleoprotein particles is revealed by *snf+* gene dosage effects. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14451–14458.

Deshpande, G., Samuels, M. E., and Schedl, P. (1996). *Sex-lethal* interacts with splicing factors in vitro and in vivo. *Mol. Cell. Biol.* **16**, 5036–5047.

Deshpande, G., Calhoun, G., and Schedl, P. (1999). The N-terminal domain of Sxl protein disrupts *Sxl* autoregulation in females and promotes female-specific splicing of *tra* in males. *Development* **126**, 2841–2853.

Graham, P., Penn, J. K. M., and Schedl, P. (2002). Masters change, slaves remain. *Bioessays* **25**, 1–4.

Horabin, J., and Schedl, P. (1993). Sex-lethal autoregulation requires multiple cis-acting elements upstream and downstream of the male exon and appears to depend largely on controlling the use of the male exon 5′ splice site. *Mol. Cell. Biol.* **13**, 7734–7746.

Lallena, M. J., Chalmers, K. J., Llamazares, S., Lamond, A. I., and Valcarcel, J. (2002). Splicing regulation at the second catalytic step by Sex-lethal involves 3′ splice site recognition by SPF45. *Cell* **109**, 285–296.

Nagengast, A. A., Stitzinger, S. M., Tseng, C., Mount, S. M., and Salz, H. K. (2003). *Sex-lethal* splicing autoregulation in vivo: Interactions between SEX-LETHAL, the U1 snRNP and U2AF underlie male exon skipping. *Development* **130**, 463–471.

Schutt, C., and Nothiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. *Development* **127**, 667–677.

Sosnowski, B. A., Belote, J. M., and McKeown, M. (1989). Sex-specific alternative splicing of RNA from the *transformer* gene results from sequence-dependent splice site blockage. *Cell* **58**, 449–459.

Yanowitz, J., Deshpande, G., Calhoun, G., and Schedl, P. (1999). An N-terminal truncation uncouples sex transforming and dosage compensation functions of *Sex-lethal*. *Mol. Cell. Biol.* **19**, 3018–3028.

## Biography

Jill Penn is a graduate student at Princeton University. She received her B.S. in Engineering Chemistry from Oakland University in Rochester, Michigan.

Patricia Graham is a post doctoral Fellow at Princeton University. She received her Ph.D. from the University of Wisconsin-Madison.

Dr. Paul Schedl is a Professor in the Department of Molecular Biology at Princeton University. He holds a Ph.D. degree from Stanford University and received his postdoctoral training at Biozentrun in Switzerland.

# Amine Oxidases

**Giovanni Floris**
*University of Cagliari, Cagliari, Italy*

**Alessandro Finazzi Agrò**
*University of Rome "Tor Vergata," Rome, Italy*

**Amine oxidases (AOs), a widespread class of enzymes, are present in all living systems, where they control the level of very active compounds, i.e., mono-, di-, and polyamines. The oxidation of these compounds may generate other biologically active substances, like aldehydes, ammonia, and hydrogen peroxide, which either directly or indirectly influence cells and tissues. Hydrogen peroxide, which is always formed in the reactions catalyzed by AOs, is more and more considered either a crucial substrate for important biochemical processes or a signal and a defense molecule, rather than a noxious waste product.**

## Classification

The oxidative deamination of mono-, di-, and polyamines is catalyzed by a number of AOs that exhibit different patterns of substrate specificity and inhibitor sensitivity and also differ in their action mechanism. Amine oxidases have been divided into two main categories, depending on the nature of the cofactor involved. One class is characterized by the presence of flavin adenine dinucleotide (FAD) as the redox cofactor. The enzymes belonging to this class are further subdivided into monoamine oxidases (MAO A and MAO B) and polyamine oxidases (PAOs). The second class is represented by enzymes having a tightly bound $Cu^{II}$ ion and a carbonyl-type group identified as 6-hydroxydopa quinone (2,4,5-trihydroxyphenethylamine quinone; TPQ) at the active site (Figures 1A and 1B). TPQ is derived from the oxidation of a tyrosyl residue in a posttranslational event. It has been proposed that the bound copper itself catalyzes the process of TPQ formation by the initial insertion of an oxygen atom into the tyrosine ring to generate dihydroxyphenylalanine (Scheme 1).

## Distribution, Reaction Mechanism, and Physiological Roles

### FAD-Containing Monoamine Oxidases

MAO A and MAO B [amine:oxygen oxidoreductase (deaminating); EC 1.4.3.4] have subunits of $M_r$ of 59.7 and 58 kDa, respectively. The crystal structure of human MAO B shows that FAD is covalently bound through the flavin C8α-position to a cysteine (Cys397) side chain (Figure 1C). MAOs are ubiquitous in the cells of most mammalian species, the notable exception being erythrocytes, and are tightly associated with the mitochondrial outer membrane, although both MAOs have been also found in the microsomal fraction. The distribution of MAOs has been studied principally in the brain: MAO A is found in catecholaminergic neurons, whereas MAO B is abundant in serotonergic and histaminergic neurons and glial cells. Moreover, some tissues mainly contain MAO A (human placenta and bovine tyroid), and other tissues contain predominantly MAO B (human platelets and bovine liver and kidney).

MAOs oxidize primary amino group of arylalkyl amines to form an imine product with the concomitant reduction of flavin to $FADH_2$. The imine is then hydrolyzed to the corresponding aldehyde and ammonia, and $FADH_2$ is oxidized back to FAD by oxygen with the formation of hydrogen peroxide. MAO A oxidizes preferentially dopamine, noradrenaline, and serotonin and is sensitive to the irreversible inhibitor clorgyline. MAO B oxidizes preferentially benzylamine and phenylethylamine and has a higher affinity for the inhibitor deprenyl. Adrenaline, kynuramine, tyramine, and tryptamine are substrates for both enzymes.

MAO A and B have been implicated in apoptosis, immunosuppression, cytotoxicity, cell growth and proliferation. These enzymes play a protective role in the body by preventing the entry of amines at the renal and intestinal levels or by oxidizing them in blood. In fact, liver MAOs are involved in controlling the blood level of pressor amines. MAO A can oxidize circulating serotonin thus preventing its effects on the heart and vascular system. Both MAO A and MAO B have important roles in the metabolism of neurotransmitters and other biogenic amines in the brain and are implicated in a large number of neurological and psychiatric disorders. The loss of dopaminergic neurons in the substantia nigra, which causes Parkinson's disease, has been associated

 © 2004, Elsevier Inc. All Rights Reserved. 

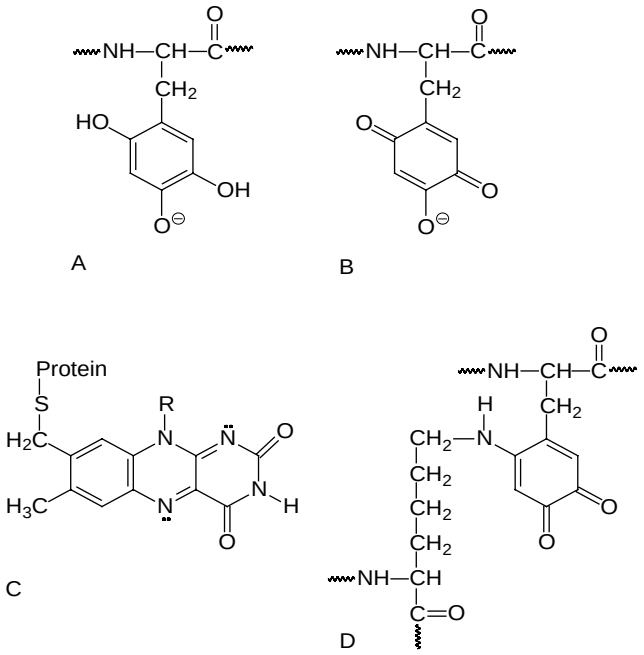
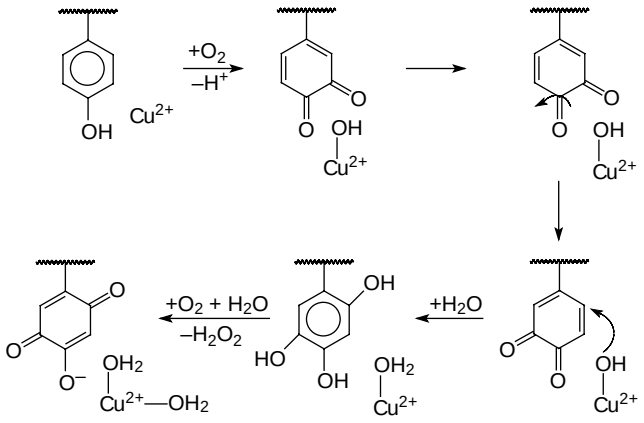



**FIGURE 1** Structure of the (A) reduced and (B) oxidized form of TPQ; (C) FAD covalently bound in MAO B; (D) lysine tyrosylquinone.

with an increased dopamine oxidation by MAO B producing high amounts of oxygen radicals responsible for oxidative damage of nigrostriatal neurons. Therefore, the pharmacological regulation of MAO activity has been shown to be important in the treatment of depression and Parkinson's disease.

## FAD-Containing Polyamine Oxidases

FAD-containing polyamine oxidases (PAOs) are monomeric enzymes with a $M_r$ ranging from 53 to 63 kDa, with 1 mol of FAD per mol of protein. The prosthetic FAD is noncovalently bound to the protein.



**SCHEME 1** Postulated pathway for the biogenesis of TPQ from tyrosine.

PAOs [$N^1$-acetylspermidine:oxidoreductase (deaminating); E.C. 1.5.3.11] are intracellular enzymes mainly found in vertebrates and plants. PAOs with characteristics similar to vertebrate enzymes are present in yeasts and amoebae, whereas bacteria and protozoans contain enzymes similar to those of plants. Plant PAOs have been isolated and characterized, particularly from the Gramineae oat, maize, barley, wheat, and rye. The enzyme from maize seedlings has been crystallized.

The mechanism by which PAOs catalyze the oxidation of polyamines is still unknown, but the catalytic mechanism can be divided into two half-reactions: (1) flavin reduction upon polyamine oxidation followed by (2) flavin reoxidation by molecular oxygen. Polyamine oxidases catalyze the oxidation of polyamines at the secondary amino group yielding different products according to the organism considered. Mammalian PAOs oxidize preferentially acetyl spermine and acetyl spermidine: spermidine and putrescine are respectively formed as reaction products, together with 3-aminopropanal and hydrogen peroxide. Oxidation of spermine by plant PAOs gives 1,3-diaminopropane, hydrogen peroxide, and 1-(3-aminopropyl)-4-aminobutanal. The latter spontaneously cyclizes to 1-(3-aminopropyl)pyrrolinium that undergoes further spontaneous rearrangements to 1,5-diazobicyclo[4.3.0.]nonane (Scheme 2). The oxidation of spermidine by plant PAOs gives 1,3-diaminopropane, hydrogen peroxide, and 4-aminobutanal that yields 1-pyrroline by spontaneous cyclization. Contrary to the reaction of MAOs and copper/TPQ AOs, the oxidation of polyamines by PAOs does not release ammonia.

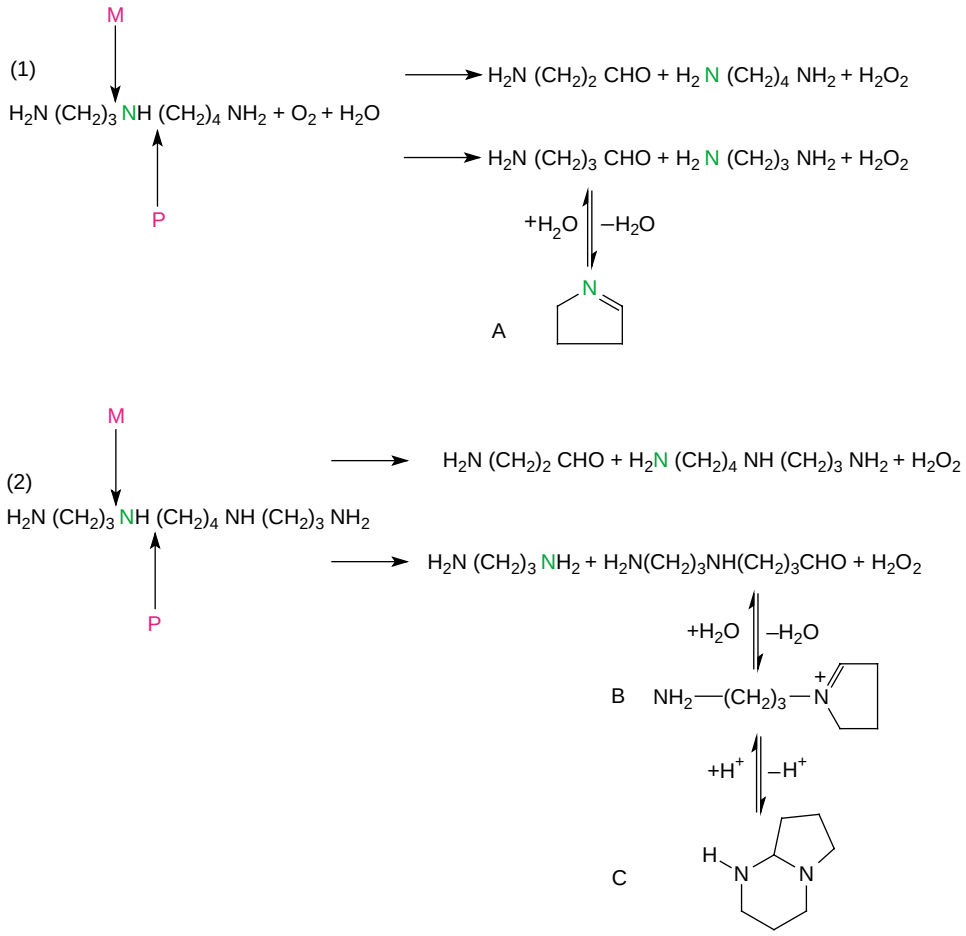
PAOs play an important role in the regulation of intracellular polyamine level, and seem to be important for homeostasis and cell survival.

## Cu/TPQ Amine Oxidases

Copper/TPQ-containing amine oxidases [amine:oxygen oxidoreductase (deaminating) (copper containing); EC 1.4.3.6] are homodimers in which each subunit ($M_r$ 70–90 kDa) contains a tightly bound type II copper ion and a quinone (TPQ). Copper/TPQ AOs catalyze the oxidative deamination of primary amino groups of mono-, di-, and polyamines, abstracting two electrons from amines and transferring them to molecular oxygen, to form the corresponding aldehyde, ammonia and hydrogen peroxide. Again, the catalytic mechanism can be divided into two half-reactions: (1) enzyme reduction at the quinone moiety (TPQ → $TPQH_2$) by substrate followed by (2) reoxidation by molecular oxygen:

$$E_{ox} + R{-}CH_2{-}NH_3^+ \rightarrow E_{red}{-}NH_3^+ + R{-}CHO \quad (1)$$

$$E_{red}{-}NH_3^+ + O_2 + H_2O \rightarrow E_{ox} + NH_4^+ + H_2O_2 \quad (2)$$




**SCHEME 2** Reaction catalyzed by mammalian (M) and plant (P) PAOs. (1) Spermidine; (2) Spermine; (A) 1-pyrroline; (B) 1-(3-aminopropyl)- pyrrolinium; and (C) 1,5-diazobicyclo[4.3.0.]nonane.

Within the class of Cu/TPQ amine oxidases are included:

(i) The intracellular amine oxidases, also called diamine oxidases (DAOs), are ubiquitous enzymes occurring in microorganisms (fungi and bacteria), plants, and mammals. Some DAOs have been crystallized from bacteria, the yeast *Hansenula polymorpha*, and from pea seedlings. The crystal structure shows that the copper atom is coordinated by three histidine side chains and two water molecules, laying at approximately 6Å distance from TPQ (Figure 2). Plant DAOs from various species have been purified to homogeneity and characterized, the best known and studied being those from lentil (*Lens esculenta*) and pea (*Pisum sativum*) and from latex of the shrub *Euphorbia characias*. In mammals, the best known enzymes are those from pig kidney and intestine, and from human placenta. DAOs prefer short aliphatic diamines like putrescine (1,4 diaminobutane) and cadaverine (1,5 diaminopentane) as substrates.

The role of DAOs is difficult to define, because this class includes several enzymes with different localizations and substrates. Bacteria and yeasts can utilize amines as nitrogen and carbon sources through the reaction with amine oxidase. Plant DAOs have an important role in cell growth by regulating the intracellular di- and polyamine levels, and the aldehyde products might have a key role in the biosynthesis of some alkaloids. The function of amine

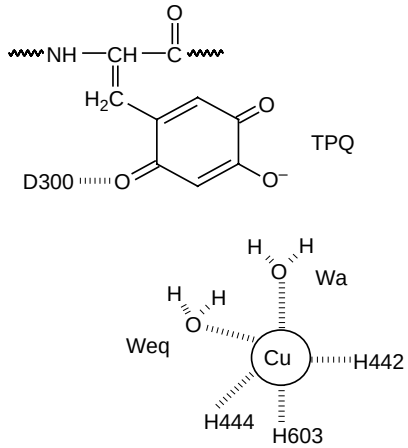



**FIGURE 2** Structural arrangement of the copper center and TPQ in the active site of pea seedling amine oxidase. D300 is the base required for the catalytic mechanism.

oxidases in mammals is even more diverse and elusive. Amine oxidase activity is found in many tissues, the highest levels being in decidual cells of placenta, in kidney tubular epithelial cells, and in intestinal epithelial cells. These localizations may suggest a general barrier function for this enzyme in preventing the entrance of extracellular diamines and polyamines into circulation. Furthermore, these enzymes may keep under control the endogenous histamine, which may be responsible for several pathological conditions like allergy, peptic ulcer, and anaphylactic reactions. Several observations point to a relationship between amine oxidase activity and growth, both in normal and tumoral tissues possibly correlated with cell proliferation and differentiation. DAOs have also been proposed as immune response modulators. It has been demonstrated that human placental diamine oxidase is identical to the amiloride-binding protein and thus in some way is involved in the regulation of epithelial ion transport.

(ii) The mammalian extracellular plasma soluble and intracellular tissue-bound amine oxidases are able to metabolize mono-, di-, and polyamines, even though *in vitro* they are preferentially active toward nonphysiological amines like benzylamine. Plasma soluble AOs are generally termed either plasma and serum AOs or benzylamine oxidases (BzAOs). Tissue-bound AOs are often indicated as semicarbazide-sensitive amine oxidases (SSAOs), somewhat misleading since all Cu/TPQ AOs, at variance with FAD-dependent enzymes, are inhibited by semicarbazide and other carbonyl reagents. There has been considerable disagreement whether the extracellular plasma AO were a product of a different gene or a cleavage product of a tissue-bound amine oxidase. The better known examples of BzAOs are those from bovine, swine, and equine plasma. Intracellular SSAOs are widely distributed: smooth muscle cells of vascular tissue of all mammalian species are a good source of these enzymes, which have been also detected in uterus, ureter and vas deferens, in ox dental pulp, in human umbilical artery, in rat adipocytes, and chondrocytes.

The increased BzAO activity in blood serum of pregnant women supports the possibility that this enzyme has a protective role against the polyamines released from the fetoplacental unit. SSAOs have been also shown to be a new class of DNA-binding proteins: in the presence of polyamines they bind DNA and oxidize DNA-bound polyamines. Structural similarity between SSAOs and VAP-1, a protein involved in cellular adhesion, has been observed. SSAOs seem to be involved in the regulation of glucose metabolism (via the $H_2O_2$ produced?) and in the regulation of leukocyte trafficking in endothelial cells.

(iii) The extracellular matrix-bound lysyl oxidase has the best-defined role: it catalyzes maturation and aging of collagen and elastin, by oxidizing the ε-aminogroups of their lysyl residues, allowing the formation of cross-links essential for the structure of these proteins. Lysyl oxidase differs from other members of the group because lysine tyrosyl quinone (Figure 1D) rather than TPQ is the redox cofactor. The genetic or acquired decrease of lysyl oxidase activity is accompanied by severe pathological conditions like the formation of aneurisms in arteries.

## Concluding Remarks

In the past few years, evidence has accumulated about the physiological relevance of hydrogen peroxide, which is generated in the catabolism of mono-, di-, and polyamines by all amine oxidases. This reactive oxygen species is toxic at high concentration. However, at lower concentrations it regulates cell number during embryonic development as well as the proliferation and adhesive properties of endothelial and smooth muscle cells. Hydrogen peroxide appears to be involved in the impairment of cell growth and proliferation, in the regulation of many genes and transcription factors, and in the transduction of cellular signals in many living species. In plants, hydrogen peroxide might be utilized by peroxidases in crucial physiological events, such as in development, in the polymerization of lignin and suberin precursors, and in the catabolism of indoleacetic acid, in response to wounding or to pathogen invasion.

### See Also the Following Articles

Flavins • Quinones

### Glossary

**amines** Hydrocarbon compounds bearing an amine group. They are called primary, secondary, or tertiary when nitrogen binds two, one, or zero hydrogen atoms.

**oxidases** Enzymes that oxidize substrates using molecular oxygen.

**polyamines** Hydrocarbon compounds bearing both primary and secondary amino groups (e.g., spermine and sperimidine) strongly interacting with nucleic acids involved in many important cellular functions.

**primary amines** Metabolically derived from amino acids by decarboxylation. Primary amines often show potent pharmacological or hormonal activity (e.g., histamine, serotonin, GABA, and noradrenaline).

### Further Reading

Binda, C., Mattevi, A., and Edmondson, D. E. (2002). Structure–function relationship in flavoenzyme-dependent amine oxidation. *J. Biol. Chem.* **277**, 23973–23976.

Federico, R., and Angelini, R. (1991). Polyamine catabolism in plants. In *Biochemistry and Physiology of Polyamines in Plants* (R. D. Slocum and H. E. Flores, eds.) pp. 41–56. CRC Press, Boca Raton, FL.

Girmen, A. S., Baenziger, J., Hotamisligil, G. S., Konradi, C., Shalish, C., Sullivan, J. L., and Breakefield, X. O. (1992). Relationship

between platelet monoamine oxidase B activity and alleles at the MAOB locus. *J. Neurochem.* **59**, 2063–2066.

Jalkanen, S., and Salmi, M. (2001). Cell surface monoamine oxidases: Enzymes in search of a function. New EMBO Member's Review. *The EMBO Journal* **20**, 3893–3901.

Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L., and Klinman, J. P. (1990). A new redox cofactor in eukaryotic enzymes: 6-hydroxydopa at the active site of bovine serum amine oxidase. *Science* **248**, 981–987.

Lyes, G. A. (1995). Substrate-specificity of mammalian tissue–bound semicarbazide–sensitive amine oxidase. In *Progress in Brain Research* (P. M. Yu, K. F. Tipton and A. A. Boulton, eds.) Vol. 106, pp. 293–303. Elsevier Science BV, Amsterdam.

Medda, R., Padiglia, A., Bellelli, A., Pedersen, J. Z., Finazzi Agrò, A., and Floris, G. (1999). $Cu^{I}$-semiquinone radical species in plant copperamine oxidases. *FEBS Lett.* **453**, 15.

Mondovì, B. (1985). *Structure and Functions of Amine Oxidases*. CRC Press, Boca Raton, FL.

Mure, M., Mills, S. A., and Klinman, J. P. (2002). Current topics. Catalytic mechanism of the Topa quinone containing copper amine oxidases. *Biochemistry* **41**, 9269–9278.

Shish, J. C., Chen, K., and Ridd, M. J. (1999). Monoamine oxidase: From genes to behavior. *Annu. Rev. Neurosci.* **22**, 197–217.

Tipton, K. F., and Strolin Benedetti, M. (2002). Amine oxidase and the metabolism of xenobiotics. In *Enzyme Systems That Metabolise Drugs and Other Xenobiotics* (C. Ioannides, ed.) pp. 95–146. Wiley & Sons, New York.

Wilce, M. C. J., Dooley, D. M., Freeman, H. C., Guss, J. M., Matsunami, H., McIntire, W. S., Ruggiero, C. E., Tanizawa, K., and Yamaguchi, H. (1997). Crystal structure of the copper-containing amine oxidase from *Arthrobacter globiformis* in the holo and apo forms: implication for the biogenesis of topaquinone. *Biochemistry* **36**, 16116–16133.

## Biography

Alessandro Finazzi Agrò is full Professor of Enzymology, Faculty of Medicine, at the University of Rome "Tor Vergata," Italy. He is currently Rector of the same University. For the past 35 years he has been studying copper proteins and copper enzymes with respect to their biochemical, physiological, and pathological roles.

Giovanni Floris is full Professor of Biochemistry and Molecular Biology, Faculty of Science, at the University of Cagliari, Italy. He is working on the purification and characterization of enzymatic proteins, particularly on plant copper/TPQ amine oxidases.

# Amino Acid Metabolism

**Luc Cynober**
*Hôtel-Dieu Hospital and Paris 5 University, Paris, France*

**Amino acids are major macronutrients involved in (1) protein synthesis, (2) energy requirements, and (3) specific functions either directly (as mediators) or through their metabolism into mediators or hormones. This article describes amino acid metabolism with special emphasis on tissue-specific metabolism, inter-organ exchanges, and regulation.**

## Structure, Functions, and Classification of Amino Acids

Amino acids (AAs) are defined as organic molecules possessing an amino moiety located at $\alpha$-position to a carboxylic group. AAs therefore have the following general formula:

$$R-\alpha CH\begin{<}NH_2\\COOH\end{>}$$

where R may be an aliphatic or a cyclic structure.

Note that proline is considered as an AA even though its $-NH_2$ group is part of a heterocycle. Also, taurine is considered as an AA even though it has a sulfur moiety instead of an amino group in the $\alpha$-position.

AAs can be classified in three different ways (Table I):

- *Chemical Classification* This classification is based on structure and identifies different chemical families of AAs: aliphatic (subdivided into several subgroups – see Table I for details), aromatic, heterocyclic, etc.
- *Metabolic Classification* Although in theory most AAs can be precursors of glucose, *in vivo*, because most AAs preferentially use other metabolic pathways, only alanine (ALA), glutamine (GLN), and to a lesser extent proline (PRO) and glycine (GLY) contribute significantly to gluconeogenesis.

Certain AAs can be precursors of ketone bodies (ketogenic AAs). Again, *in vivo*, only leucine (LEU) contributes significantly to ketogenesis.

Finally, some AAs can be both glucogenic and ketogenic: isoleucine (ILE) and phenylalanine (PHE).

- *Nutritional Classification* This is based on what AAs the human body can or cannot synthesize. The former are named nonessential AAs (NEAAs) and the latter essential AAs (EAAs). There are nine EAAs (Table I). Note that some NEAAs can become EAAs in specific situations, e.g., arginine (ARG) during growth, tyrosine (TYR) during renal failure, or GLN in trauma patients.

## Intestinal Absorption of AAs

Digestion of proteins (beyond the scope of this chapter) releases a mixture of free AAs and short-chain peptides. Nitrogen is absorbed mainly in the jejunum and the ileum in the form of free AAs and di- and tripeptides. It is now well recognized that the latter have a kinetic advantage for uptake. To date, two peptide transporters have been cloned, PEPT-1 and PEPT-2.

AAs are taken up by systems that are rather different from those found in other cells and also from those located at the basolateral side of enterocytes. The groups of transporters are relatively specific to:

- neutral AAs,
- imino acids,
- dibasic AAs + cysteine (CYS), and
- dicarboxylic AAs.

## Intestinal Metabolism

It was long thought that the gut had a single function, namely, taking up AAs from the lumen for transport in the bloodstream.

In fact, the intestine avidly consumes some AAs for its energy requirements: GLU and GLN are used to the same extent as glucose by enterocytes, producing $\alpha$-ketoglutarate, which is oxidized in the Krebs cycle. After a balanced meal, at least 30% of GLU + GLN is used by enterocytes, and as a consequence ammonia is released in the portal vein. Interestingly, when GLN is taken up at the luminal side of enterocytes, its uptake at the basolateral side decreases. The reverse is true in the postabsorptive state, so that the supply of GLN to enterocytes remains roughly constant.

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Classifications of Amino Acids.**

| Aliphatic | Aromatic |
|---|---|
| • Short-chain | ***Phenylalanine*** |
| **Glycine** | **Tyrosine** |
| **Alanine** | Heterocyclic |
| • Alcohol | *Tryptophan* |
| ***Threonine*** | Dibasic |
| **Serine** | **Arginine** |
| • Branched-chain | *Histidine* |
| *Leucine* | *Lysine* |
| ***Isoleucine*** | Diacid |
| *Valine* | **Glutamate** |
| • Sulfur | **Glutamine** |
| *Methionine* | **Aspartate** |
| Cysteine | **Asparagine** |
| Imino acid | |
| **Proline** | |

Only AAS engaged in protein bonds are presented. Note that there are numerous other AAs: (1) intermediary metabolites: ornithine, citrulline; and (2) posttranscriptionally formed: hydroxyproline, γ-carboxyGLU acid – (a) in italics: essentials AAs; (b) in bold type: gluconeogenic AAs; and (c) underlined; ketogenic AAs.

Also, the turnover of enterocytes is very rapid, causing a strong requirement for purine and pyrimidine precursors (e.g., GLN).

## Cellular Transport of AAs

Once AAs appear in the circulation, cellular transport is a critical step in AA metabolism, since it is a prerequisite for any further metabolism. In certain cases this step may even be rate limiting or rate controlling for metabolic pathways.

The systems of transport have long been classified according to their preferred substrates, their dependency towards sodium, their sensitivity to pH, and their ability to transport nonmetabolizable analogues. Based on these criteria a large number of transport systems have been identified. The most ubiquitous are system A (A for ALA-preferring), system ASC (ALA-, serine (SER-) and CYS-preferring), system L (LEU-preferring), y + (ARG being the main substrate), N (for the transport of GLN and histidine (HIS)), etc. With progress in molecular biology methods, a number of transporters have been cloned, allowing a new classification. Not surprisingly, the number of transport systems was found to be greater. For example, for ARG, four transporters have been cloned (CAT-1, CAT-2A, CAT-2B, and CAT-3) with different cell localizations, properties, and affinities for the different cationic AAs.

The organs that are most contributive to AA metabolism are the liver and muscle.

**TABLE II**

**Some Amino Acid Functions**

| Function | Amino acid | Example |
|---|---|---|
| Constituent of proteins | 20 AAs | |
| Hormone precursor | Phenylalanine | Tyroxine |
| | Tyrosine | Cathecholamines |
| | Tryptophan | Serotonin |
| Mediators | Glutamate | Glutamate, GABA |
| | Glutamine | Glutamine |
| | Arginine | Nitric oxide |
| Binding of calcium | Glutamate | γ-carboxyglutamate |
| Collagen structure | Proline | hydroxyproline |

GABA: γ-aminobutyric acid.

## Liver Metabolism

The liver holds a central place in the metabolism of AAs because it is responsible for the synthesis of most circulating proteins, transforms AAs as energy sources (i.e., glucose and ketone bodies) for other tissues, and eliminates surplus nitrogen.

AAs supplied via the portal vein after a meal are heavily metabolized (~50%). The carbon chain is oxidized or forms glucose, which is saved as glycogen, while the N-moiety (ies) is (are) removed in ureagenesis.

The reason for this process is that whereas the intestinal absorption of AAs is not limiting (95–99% of nitrogen is absorbed in a large range of protein intake), the brain must be protected against excessive AA exposure because several of them are neurotoxic or are the precursors of potent neuromediators (see Table II). Thus, the liver acts as a filter, limiting the amount of AAs released in the general circulation. Notable exceptions are the branched-chain AAs (BCAAs; VAL, ILE, LEU), which are almost nonmetabolized in the liver: BCAAs form ~22% of AAs in food proteins but almost 50% of AAs reaching the general circulation.

The biochemical explanation for this effect is that hepatocytes do not contain BCAA transaminase, which mediates the first step of BCAA metabolism. The physiological reason may be related to the fact that LEU plays a critical role in the stimulation of muscle protein synthesis in the postprandial phase.

In the postabsorptive state, with glycogen exhaustion, gluconeogenesis increases to maintain glucose homeostasis. As stated above, ALA is the main gluconeogenic

substrate among AAs: in healthy humans starved for 8 h, 30% of perfused ALA is metabolized into glucose, and, in these conditions, 11% of glucose formed by the liver comes from ALA. When the starvation time is increased, and in situations of hyperglucagonemia (e.g., during response to injury such as burn, trauma, or sepsis), the contribution of ALA to gluconeogenesis increases like those of other AAs, in particular GLN. In these situations, most AAs taken up by the liver come from the muscles.

During starvation, another metabolic pathway is activated: ketogenesis is mainly supported by free fatty acid metabolism, but one AA, LEU, is also contributive. This may seem paradoxical because, as stated above, hepatocytes do not express BCAA-T. However, hepatocytes express all the other enzymes required for ketone body (KB) production, in particular the highly regulated enzyme branched-chain keto-acid-dehydrogenase (BCKA-dh). Thus, as described below, the production of KB from LEU is a typical example of the importance of inter-organ exchanges in AA metabolism.

## Muscle Metabolism

Muscles contain ~50% of the proteins in the human body and the largest pool of free AAs (i.e., 87 g in a male weighing 70 kg; by comparison the plasma pool is only 1.2 g).

Therefore, muscles can be considered as a reserve of AAs, either directly (i.e., as free AAs) or indirectly (i.e., in the form of proteins).

After a meal, arterio-venous measurements indicate that all AAs are taken up by the muscles to support protein synthesis.

At the postabsorptive state, all but one (GLU) are released by the muscles. It is noteworthy that ALA + GLN together represent 60% of the total AAs released by muscle, whereas they form less than 20% of protein content. This underlies the fact that most ALA and GLN comes from *de novo* synthesis in the muscle. The carbon chain required for ALA synthesis comes from anaerobic degradation of glucose, and the amino moiety comes from transamination with GLU. The resulting $\alpha$-KG is retransaminated by BCAA-T releasing BCKAs from BCAAs (Figure 1). Ultimately, 18% of the glucose taken up by muscle is metabolized into ALA and 12.5% of the nitrogen contained in circulating ALA comes from LEU. For GLN, the carbon chain comes from GLU and GLU comes both from transamination and from the bloodstream. The ammonia required for the amidation of GLU comes from oxidative deamination of AAs and from the degradation of purine bases (Figure 1).

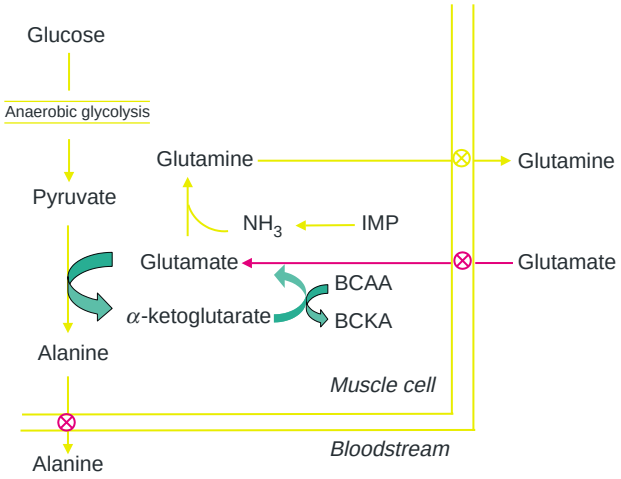



**FIGURE 1** *De novo* synthesis of alanine and glutamine in muscles at the postabsorptive state. IMP, inosin monophosphate; BCAA, branched-chain amino acid; BCKA, branched-chain keto acid.

## Inter-Organ Exchanges

Every tissue or organ possesses enzymatic equipment that is specific both qualitatively and quantitatively. Correspondingly, each tissue plays a specific role in nitrogen homeostasis. This explains the importance of inter-organ exchanges, each organ contributing as a provider of those AAs that others are unable (or not sufficiently able) to produce. The inter-organ exchanges are highly dependent on the feeding state (i.e., postprandial versus postabsorptive).

At the postprandial state, as mentioned above, there is major AA splanchnic sequestration. AAs appearing in the general circulation are taken up by peripheral tissues, especially muscles.

At the postabsorptive state, the body must use its stores to generate the energy required. Lipolysis and glycogenolysis are contributive to this process. However, the glycogen stores are limited and as fasting progresses, the glucose supply becomes more and more dependent on gluconeogenesis. This process implies the transfer to the liver of gluconeogenic AAs such as ALA and GLN. As described earlier in this article, the nitrogen moiety ultimately comes from BCAAs. This fact has two consequences:

1. Since BCAAs are essential AAs (i.e., no possibility of synthesis in humans), their sole source is protein breakdown. Hence gluconeogenesis in the liver results in net protein breakdown in muscle.
2. Branched-chain keto acids (BCKAs) resulting from BCAA transamination are poorly metabolized in muscles (except in severe catabolic situations where BCKA-dh is activated by proinflammatory cytokines)

and therefore released in the bloodstream. They are then taken up by the liver, where two of them ($\alpha$-ketoisocaproate and $\alpha$-ketomethylvalerate) contribute to ketogenesis.

Gluconeogenic AAs taken up by the liver are readily transformed into glucose and this is favored by increased glucagon levels and decreased insulin secretion. Of course, synthesis of one molecule of glucose from one molecule of AA requires the removal of the N-residues. This is the job of ureagenesis. This process has an important consequence: each molecule of glucose produced in this pathway leads to the irreversible loss of (at least) one N-residue from net muscle proteolysis. This explains why in conditions of prolonged food restriction or when energy demand increases (e.g., in injury), a characteristic sign of metabolic adaptation is loss of muscle mass.

Glucose produced by the liver is taken up by glucose-dependent tissues. In the muscle, glucose is largely used anaerobically, leading to further generation of alanine, producing an alanine–glucose–alanine cycle also called the Cahill cycle.

At the whole-body level, this cycle makes no sense energetically because anaerobic glucose consumption generates little ATP and gluconeogenesis consumes at least the same amount of ATP. However, reasoning at the tissue level casts a different light: in the postabsorptive state the liver is rich in energy owing to $\beta$-oxidation of free fatty acids from lipolysis in adipose tissue, whereas muscle is somewhat depleted in energy. Therefore, the Cahill cycle corresponds ultimately to the transfer of energy from an organ that is energy-rich (the liver) to a tissue that demands energy (muscle) at the cost of net protein breakdown.

## Final Products of AA Metabolism, Elimination of Surplus Nitrogen

N-metabolic products of AAs are mostly excreted in urine. Only an average 7 mg $kg^{-1}$ body weight in males and 8 mg $kg^{-1}$ in females is removed daily in other ways: shed skin (5 mg $kg^{-1}$), nasal secretions, shed hair, menstrual losses, etc.

Elimination of nitrogen cannot be carried out directly as ammonia because this substance is toxic for the central nervous system at plasma concentrations above 50 $\mu$mol $l^{-1}$. Yet the equivalent of a mole of $NH_3$ has to be cleared every day. For this reason, in humans, the main form of nitrogen elimination is urea, a water-soluble nontoxic compound synthesized by the liver.

However, not all the ammonia must be converted into urea, because ammonia plays a key role in acid–base homeostasis, in particular in the kidney.

### Ureagenesis

The urea cycle is partly cytoplasmic and partly mitochondrial. Only the liver possesses all the enzymes required to synthesize urea from ammonia, and this pathway is strictly located in periportal hepatocytes.

Five enzymes are involved: carbamoylphosphate synthase (CPS), ornithine carbamoyltransferase (OCT), arginosuccinate synthase, arginosuccinate lyase, and arginase.

Ureagenesis is governed by three different types of regulation:

1. Regulation by the availability of precursors: the rate of flux of AAs towards the liver is a key regulator of ureagenesis; the more AAs the liver takes up, the higher is the rate of ureagenesis. AAs may come from food in the postprandial phase or from muscles in the post-absorptive phase. Conversely, during prolonged starvation, urea production declines simply because the muscle efflux of AAs decreases. All the AAs are not equally ureogenic: GLN, ALA, and ARG are the most contributive.
   - GLN is hydrolyzed into GLU and ammonia by glutaminase present in large amounts in periportal hepatocytes. This reaction forms a ureagenesis amplification loop because the product of the reaction (ammonia) has the unusual property of activating glutaminase. The accumulation of GLU, the other product of the reaction, allows the synthesis of N-acetylglutamate, the regulatory role of which is described next.
   - ALA is transaminated into PYR and in parallel $\alpha$-ketoglutarate gives rise to GLU. This GLU forms a pool distinct from those described above: a second transamination reaction turns oxaloacetate into aspartate, which provides the second nitrogen donor in ureagenesis.
   - ARG is very ureagenic because (1) it is the direct precursor of urea and (2) it plays a key role in the allosteric regulation of ureagenesis.
2. Allosteric regulation: role of N-acetylglutamate. This substance plays a key role in ureagenesis regulation because it is the allosteric regulator of CPS, the enzyme controlling the entry of ammonia into the cycle. N-acetylglutamate synthesis is catalyzed by N-acetylglutamate synthase, which is strongly activated by ARG. Hence the flux of substrates (GLN, $NH_3$, and ARG) and the allosteric regulation of CPS act synergistically to modulate ureagenesis both upstream and downstream.

3. *Hormonal Regulation* This regulation operates at two levels:

- On substrate availability: cortisol increases proteolysis and muscle efflux of AAs; glucagon promotes their transport into hepatocytes and further metabolism into ureagenesis and gluconeogenesis.
- On the activity of enzymes.

### Ammoniagenesis

This pathway is located in kidney tubular cells and is 80% supported by GLN. The first step is mediated by type I phosphodependent glutaminase, an enzyme activated by acidosis; GLU can then be transaminated into $\alpha$-ketoglutarate or deaminated by GLU dehydrogenase. This last reaction is strongly activated by acidosis, further increasing the flux of $NH_3$. $NH_3$ passes freely into the lumen, where it combines with protons to form the ammonium ion, which cannot return to cells.

The one-way flux of $NH_3$ means that during acidosis (i.e., high amount of $H^+$ in the lumen) the intra-cellular $NH_3$ level is low, derepressing GLU-dh. In turn, this favors GLU metabolism and low GLU derepresses glutaminase. Hence it appears that ammoniagenesis is an adaptative pathway that plays a fundamental role in metabolic acidosis.

### Relationship between Ureagenesis and Ammoniagenesis: A Contribution to Acid–Base Homeostasis

The observations described above underline the unique role of GLN as a donor of nitrogen for both ureagenesis and ammoniagenesis. However, heavy consumption of GLN in ureagenesis is not compatible with an increased demand by the kidney in acidosis. A balance between these two almost exclusive processes is achieved, thanks to an anatomical detail – the liver contains two different hepatocyte populations:

- Periportal hepatocytes (93% of the total), which possess a glutaminase activity and enzymes of the urea cycle.
- Perivenous hepatocytes, which form only 7% of the total but have a metabolic activity 100 times higher. These cells possess GLN synthase activity.

Hence catabolism and synthesis of GLN are two processes that occur simultaneously in the liver, but at a different rates according to the situation.

- Physiologically, most of the GLN from the portal vein is taken up by periportal hepatocytes, and the liver is a net consumer of GLN.
- In acidosis, hepatic glutaminase is inhibited (note: acidosis activates kidney glutaminase and inhibits the liver isoform). Consequently, GLN remains available for amino acid metabolism.

## Hormonal Control of Amino Acid Metabolism

Physiologically as well as in disease, hormones play a key role in the control of AA metabolism, with a balance between anabolic and catabolic hormones.

### Anabolic Hormones

1. *Insulin* Insulin exerts actions at every level of AA metabolism:
   (a) It increases the cell transport of numerous AAs, especially in muscle and liver.
   (b) It favors net protein anabolism by decreasing protein breakdown.
   (c) It decreases gluconeogenesis both by decreasing the availability of precursors and by inhibiting key enzymes of this pathway.

2. *Growth Hormone (GH)* GH stimulates protein synthesis.

### Catabolic Hormones

1. *Glucagon* Like insulin, glucagon activates the A system of AA transport, but unlike insulin, glucagon favors the use of AAs in gluconeogenesis.

In addition, glucagon favors proteolysis (through macro-autophagy) in the liver.

2. *Cortisol* Cortisol induces hyper-amino-acidemia because although it increases hepatic, intestinal and renal uptake of AAs, it increases their muscle release even more strongly. In addition, cortisol favors net protein breakdown. Hence in a stress situation, cortisol and glucagon have a synergistic action (Figure 2) leading to a unidirectional flux of nitrogen from the muscle to the liver.

3. *Cytokines* Physiologically, their role is minor. However, in disease, proinflammatory cytokines (e.g., tumor necrosis factor $\alpha$, interleukins 1 and 6) are overproduced and act synergistically with glucagon and cortisol on AA metabolism.

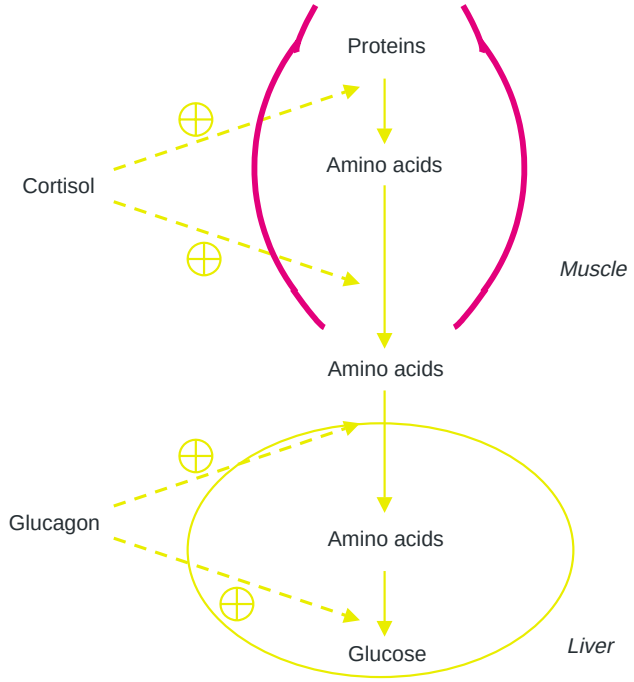



**FIGURE 2** Cortisol and glucagon act synergistically to drive AAs from muscle to the liver.

## See Also the Following Articles

Glucagon Family of Peptides and their Receptors • Gluconeogenesis • Glycogen Metabolism • $G_q$ Family • Insulin- and Glucagon- Secreting Cells of the Pancreas • Ketogenesis • Steroid/Thyroid Hormone Receptors • Urea Cycle, Inborn Defects of

## Glossary

**ammoniagenesis** *De novo* ammonia synthesis from amino acids. The main precursor is glutamine and this process occurs mainly in the kidney. Ammoniagenesis plays a key role in acid–base homeostasis.

**enterocyte** A cell that ensures the transport of nutrients from the gut lumen to the bloodstream and protects the internal milieu from invasion by bacteria and others.

**essential amino acid** An amino acid that cannot be synthesized by humans; hence, their provision is strictly dependent upon alimentation.

**gluconeogenesis** Glucose synthesis from nonglucidic precursors. Main substrates are AAs (mainly alanine, glutamine, and proline), lactate, pyruvate, and glycerol. Gluconeogenesis occurs during fasting mainly in the liver and also in the kidney.

**ketogenesis** Synthesis of ketone bodies. Fatty acids are the main substrates. Some AAs are also involved, especially leucine. Ketogenesis occurs specifically in the liver.

**ureagenesis** Synthesis of urea from ammonia or from ammonia derived from amino acids. Ureagenesis allows removal of amino acid in excess and/or the incorporation of the carbon moiety of AAs into glucose (i.e., gluconeogenesis). Ureagenesis is located in the liver.

## Further Reading

Cynober, L. A. (2002). Plasma amino acid levels with a note on membrane transport: Characteristics, regulation, and metabolic significance. *Nutrition* **18**, 761–766.

Cynober, L. (ed.) (2004). *Metabolic and Therapeutic Aspects of Amino Acids in Clinical Nutrition*. 746p. CRC Press, Boca Raton.

Felig, P. (1975). Amino acid metabolism in man. *Annu. Rev. Biochem.* **44**, 933–955.

Harper, A. E., Miller, R. H., and Block, K. P. (1984). Branched-chain amino acid metabolism. *Annu. Rev. Nutr.* **4**, 409–454.

Haussinger, D. (1990). Nitrogen metabolism in liver: Structural and functional organization and physiological relevance. *Biochem. J.* **267**, 281–290.

Husson, A., Brasse-Lagnel, C., Fairand, A., Renouf, S., and Lavoinne, A. (2003). Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. *Eur. J. Biochem.* **270**, 1887–1899.

Meijer, A. J., Lamers, W. H., and Chamuleau, R. A. (1990). Nitrogen metabolism and ornithine cycle function. *Physiol. Rev.* **70**, 701–748.

Millward, D. J. (1990). The hormonal control of protein turnover. *Clin. Nutr.* **9**, 115–126.

## Biography

Luc A. Cynober is Head of the Department of Clinical Biochemistry, Hôtel-Dieu Hospital, Paris, and Professor of Nutrition and Head of the Biological Nutrition Laboratory at the School of Pharmacy, University Paris 5. He holds a Pharm.D. and a Ph.D. from Paris XI University. Dr. Cynober is editor of *Current Opinion in Clinical Nutrition and Metabolic Care* and has chaired since 2001 the French-Speaking Society of Enteral and Parenteral Nutrition. His major research interests relate to amino acid metabolism and therapy in critical illness and aging.

# Aminopeptidases

**Ralph A. Bradshaw**
*University of California, Irvine, California, USA*

**Aminopeptidases, which are widely distributed in nature, are one of the two major subclasses of the exopeptidases, proteolytic enzymes that remove amino acids from the termini of peptides and proteins (the other being the carboxypeptidases). As the name indicates, the aminopeptidases attack their substrates exclusively from the amino terminal end. Most remove one amino acid at a time, but a small group cleaves two or three residues at a time; these are known as dipeptidyl and tripeptidyl aminopeptidases, respectively. A few enzymes such as acylaminoacyl-peptidase and pyroglutamyl-peptidase remove derivatized amino acids, but generally aminopeptidases require an unmodified or free amino group.**

## General Description

### Properties

Aminopeptidases occur in both soluble and membrane-bound forms and can be found in various cellular compartments as well as in the extracellular environment. The majority are metalloenzymes; that is, they minimally require a metal cofactor at the catalytic site for activity, which is usually zinc ion but can be a number of other metal ions, including $Fe^{2+}$, $Mn^{2+}$, and $Co^{2+}$. There are subclasses containing one and two metal ions. In some cases, the physiological relevant metal is uncertain. There are a few aminopeptidases, particularly of the dipeptidyl- and tripeptidyl-peptidase type, that are classified as serine or cysteine proteases. Aminopeptidases can be processive, meaning that they will continue to degrade the substrate until they reach an unfavorable residue (or combination of residues), and nonprocessive. The latter are usually highly specific for an amino acid type and do not further degrade the substrate after the initial susceptible residue is removed.

### Functions

The physiologic functions of aminopeptidases can be divided into three main categories: processing/maturation, activation, and degradation. The enzymes involved in the first two areas usually are highly specific, are nonprocessive, and tailor the N terminus of proteins or peptides either co- or posttranslationally to induce activity or to allow for subsequent modifications that will, in turn, affect activity. Degradation (including inactivation) can also use specific enzymes, particularly where individual bioactive peptides are targeted, but most often involves nonspecific enzymes that participate in protein turnover by the reduction of small peptides, arising from proteosomal cleavage of targeted proteins, to amino acids or by the extracellular degradation of peptides arising from a number of sources.

## N-Terminal Cotranslational Processing

One of the best understood functions of aminopeptidases is their role in N-terminal cotranslational processing. Protein synthesis that is directed by the genetic material is universally initiated by the amino acid methionine (in prokaryotes, N-formyl methionine), but the majority of the protein mass in organisms does not reflect this event; that is, most proteins (at least those commonly studied to date) do not have an N-terminal methionine residue. Extracellular proteins that are exported through the endoplasmic reticulum and proteins imported into mitochondria (as well as related organelles such as chloroplasts) lose their respective signal peptides (usually ~20–25 amino acids), including the initiator methionine, through the action of specific signal peptidases, which are endopeptidases. The removal of the initiator methionine from intracellular proteins is accomplished through the action of a specific class of aminopeptidases (clan MG of the metallopeptidases) designated methionine aminopeptidases (MetAPs). All living organisms apparently have at least one form of this enzyme, and they require this activity for vitality. There are several reasons for this: (1) methionine is a relatively scarce amino acid and failure to return a large percentage of the methionine used in the initiation process for reuse (in a variety of activities) leads to a starvation condition that is ultimately lethal; (2) many proteins are subsequently modified on the $\alpha$-amino group of the newly exposed residue (but can also occur on methionine residues that are not removed) that in many cases is a requirement for their further function; and (3) a few proteins use the newly exposed penultimate residue as a

 © 2004, Elsevier Inc. All Rights Reserved. 

part of their active structure. The reason that methionine is not removed from all N termini is apparently due to its role as a protecting group against degradation by the N-end rule pathway.

## MetAP Specificity

The substrate profile of the MetAPs, despite other distinguishing physical characteristics, is highly conserved over all living organisms, suggesting that it is a very ancient enzyme. Briefly, susceptible substrates have the seven smallest amino acids (glycine, alanine, serine, threonine, cysteine, proline, and valine) in the adjacent (penultimate) position to the methionine. In yeast, a little over one-half of the open reading frames (ORFs) are predicted to code for proteins with these N-terminal sequences, but in terms of mass the percentage of soluble proteins is much higher, perhaps as high as 80%. Because essentially all exported and transmembrane proteins (which may account for as much as one-third of the total protein in a cell) and most of the proteins imported into the mitochondrion also lose their N termini through cleavage of their signal peptides, which are in turn degraded to amino acids, there is a very high degree of recyclization of initiator methionine.

## Properties of MetAPs

### Structural Organization

The basic catalytic domain, first defined by X-ray analyses of the *E. coli* enzyme, is ~30 kDa in mass and contains two metal ions at the active site. It has an internal symmetry (described as a pita bread fold) suggestive of an early gene duplication event. This structure appears, with only minor variations, to be found in all eubacteria and represents the type 1 isoform. Archaebacteria contain a different but related (homologous) form of MetAP, principally characterized by an insertion of approximately 65 residues, that forms an extra highly helical domain on the surface of the protein. It is designated type 2. Eukaryotes contain both types and in addition each contains an amino terminal extension. The N-terminal segment of the type 1 enzyme contains zinc-finger domains that are thought to act as a tether to tie this enzyme to the ribosome. The corresponding segment of the eukaryotic type 2 proteins is marked by extended stretches of polyacidic and basic amino acids. The function of this domain is unknown. The overall organization of the MetAP family is shown in Figure 1.

### Metal Use

These enzymes were initially characterized as using two $Co^{2+}$, based on early experiments with the eubacterial MetAPs. Subsequently, it was shown that multiple metals could be used in many of the forms, particularly the *E. coli* enzyme, which is active with $Fe^{2+}$, $Zn^{2+}$, $Ni^{2+}$, and $Mn^{2+}$, in addition to $Co^{2+}$. It is possible that, depending on availability, the eubacterial enzymes may use different metals. In recent studies in eukaryotes, $Mn^{2+}$ has emerged as the preferred candidate for the type 2 enzyme, whereas $Zn^{2+}$ remains the most likely metal cofactor for the type 1 enzymes under physiological conditions. Although early studies suggested that the MetAPs used a bivalent metal structure, they can clearly function with only a single metal ion present.

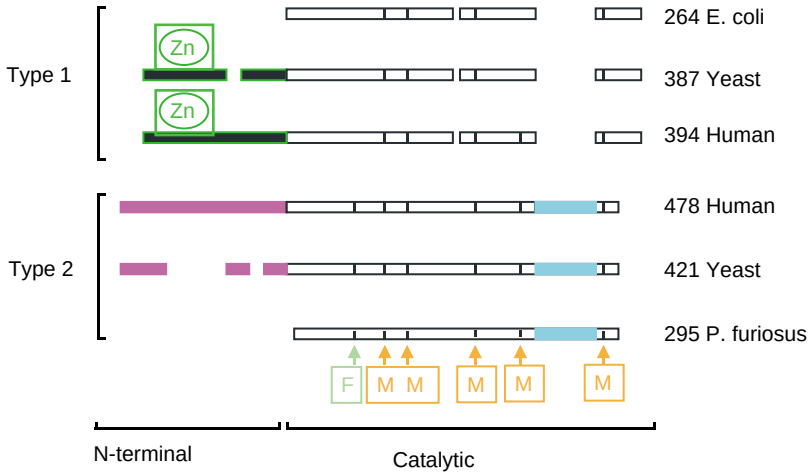



**FIGURE 1** Schematic presentation of the structural organization of the MetAP family. Separate domains are indicated by color (magenta, N-terminal extension characteristic of MetAP2s; brown, N-terminal extension with putative Zn-binding domains characteristic of MetAP1s; light blue, catalytic domain insert characteristic of MetAP2s) and are presented approximately to scale. Major deletions (and insertions) deduced from sequence and structural comparisons are indicated as gaps. M denotes the site of a metal ligand; F indicates the location of the histidine modified in type 2 enzymes. The number of residues for each protein is given in the column at the right. Reproduced with permission from Bradshaw and Yi, 2002, *Essays in Biochemistry*, Vol. 38, pp. 65–77. © the Biochemical Society.

In all cases, the metal ions are bound through five amino acid side chains and these are well preserved in all the isoforms in both prokaryotes and eukaryotes.

## Roles of the MetAP Isoforms

The substrate specificity of all of the isoforms is generally the same as just described and is heavily predicated on the nature of the penultimate residue. Substrate length, in *in vitro* studies, does have some effect but it is unclear whether this is of significance *in vivo*. Similarly, different metal ions could also affect substrate selection, but this has not been systematically demonstrated. Null mutations (manipulations that prevent the expression of a gene) in yeast demonstrate some measure of redundancy because cells will survive with one or the other of the isoforms but not when both are eliminated. Deletion of the single gene in prokaryotes is also lethal. Nonetheless, there is both direct and indirect evidence to support the view that the two isoforms have different cellular functions. MetAP1 is thought to function as the main processing enzyme and be physically associated with the ribosome in a position to hydrolyze the methionine from germane nascent chains during protein synthesis. MetAP2 is thought to be a soluble enzyme and to probably provide secondary processing for substrates that improperly escape the action of MetAP1. However, it is clearly involved in other activities as well. This is illustrated by a class of irreversible chemical inhibitors that are highly selective for MetAP2, which cause cell-cycle arrest in endothelial cells (but not cell death) resulting in anti-angiogenesis. These potential drugs are being refined for use in treating tumors. Presumably this inhibition results from the failure of MetAP2 to process a select protein or subset of proteins involved in mitosis of these cells, but their nature is unknown. Downstream sequences probably are responsible for rendering these select targets susceptible to MetAP2 but not MetAP1. MetAP2 also functions to inhibit the phosphorylation of eIF2$\alpha$ and thereby promote translation. This activity is entirely distinct from its catalytic one and is not connected to its cell-cycle functions because the inhibited protein is still fully functional as a phosphorylation inhibitor. It is thus one of many proteins known to have dual (and often unrelated) functions.

## See Also the Following Articles

Amino Acid Metabolism • Metalloproteases • N-End Rule • Proteasomes, Overview • Protein Import into Mitochondria • Zinc Fingers

## Glossary

**anti-angiogenesis** The process by which blood vessel formation is inhibited. Disruption of this activity is an effective way to prevent tissue proliferation, as is encountered in tumor growth.

**carboxypeptidase** One of two major classes of exopeptidases. They cleave protein and peptide substrates sequentially from the carboxyl terminal end.

**endopeptidase** One of two major classes of proteolytic enzymes (the other being exopeptidases) that cleave peptide and protein substrates at internal peptide bonds.

**N-end rule pathway** An intracellular pathway in which selected N termini of proteins are recognized by a specific part of the ubiquitin tagging machinery of the cell, leading to their degradation via proteosomal cleavage.

**open reading frames (ORFs)** Genome sequences that can be continuously interpreted in an unbroken protein sequence. They generally, but not always, correspond to true structural genes.

**proteosome** An intracellular suborganelle (or protein machine) composed of multiple subunits organized in a stack of four 7-membered rings. The subunits of the inner rings are proteolytic enzymes that degrade target proteins into short peptides, which are then either further broken down into free amino acids by exopeptidases action or (in the immune system) presented as cell surface antigens to elicit an antibody response. When associated with additional subunits that recognize appropriately marked proteins, it is the principal entity responsible for intracellular protein turnover.

**zinc-finger domains** Short amino acid sequences that contain four appropriately spaced residues capable of binding a zinc ion through their side chains. This structure, usually predictable from sequence alignments, is often involved in binding to both protein and nucleic acid partners.

## Further Reading

Barrett, A. J., Rawlings, N. D., and Woessner, J. F. (eds.) (1998). *Handbook of Proteolytic Enzymes*. Academic Press, London.

Bradshaw, R. A., Hope, C. J., Yi, E., and Walker, K. W. (2001). Co- and posttranslational processing: The removal of methionine. *Enzymes* **22**, 387–420.

Bradshaw, R. A., and Yi, E. (2002). Methionine aminopeptidase and angiogenesis. *Essays Biochem.* **38**, 65–78.

Lowther, W. T., and Matthews, B. W. (2000). Structure and function of the methionine aminopeptidases. *Biochim. Biophys. Acta* **1477**, 157–167.

Taylor, A. (ed.) (1996). *Aminopeptidases*. R.G. Landes Co., Austin, TX.

## Biography

Dr. Ralph A. Bradshaw is a Professor of Physiology and Biophysics at the University of California, Irvine. His principal research interests are in protein chemistry and proteomics, with a focus on N-terminal processing of proteins and protein turnover and on signal transduction by growth factors and their receptors. He received his Ph.D. degree from Duke University and was a researcher and faculty member at Indiana University, University of Washington, and Washington University.

# Amyloid

**Ronald Wetzel**
*University of Tennessee, Knoxville, Tennessee, USA*

**Amyloid is an aggregated protein structure consisting of unbranched microscopic fibrils often found in dense tissue deposits and associated with a variety of human diseases, including a number of significant neurodegenerative disorders. In its broadest usage, the term amyloid does not pertain to a specific protein molecule or sequence, but rather to a general folding pattern, or folding motif, that appears to be accessible to many, if not all, polypeptide chains. Although the three-dimensional structures of these proteins in their native states can vary enormously, the abnormal amyloid structures that they form exhibit a characteristic folding pattern, called a "cross-$\beta$" structure, that differs from that of the native state structure.**

## Introduction

Amyloid formation thus involves a protein misfolding reaction. Amyloid fibrils are generally very stable and quite insoluble in native, aqueous buffer. In a way, amyloid is a foreign substance composed of self-proteins, but it is not easily recognized and removed as a foreign substance by the immune system, for reasons that are not well understood. In some disease states involving amyloid deposition, the amyloid deposit is directly involved in the disease mechanism, whereas in other cases its mechanistic role is less clear. In some microorganisms, specific proteins and protein domains appear to have evolved for the purpose of making amyloid fibrils that play an important, positive role in the cell. In most cases, however, amyloid is a pathogenic structure, formed by accident under conditions of molecular, cellular, or organismic stress, from proteins that evolved to fold and function in quite different structural states. The recognition that proteins not known to be involved in amyloid disease can be induced to form amyloid fibrils, through exposure to certain nonnative conditions *in vitro*, has led to a picture of the amyloid motif as a general default structure in protein folding accessible to many, if not all, polypeptide sequences. Notwithstanding the predominant usage described above, the word "amyloid" is also occasionally found as part of the names of specific proteins. These proteins tend to be associated in some way with the amyloid phenomenon, either as proteins capable of making amyloid fibrils [such as serum amyloid A, islet amyloid polypeptide, and $\beta$-amyloid (also known as A$\beta$)] or as proteins that interact with amyloid fibrils (such as serum amyloid P component).

## History

Waxy masses found at autopsy in the liver and spleen have been observed in humans since the 17th century. In 1854, Virchow reported that an iodine stain thought to be specific for starch generated a positive test on such material and therefore classified these structures as amyloid (starch-like). Although 5 years later Friedreich and Kekule demonstrated that these deposits contained negligible carbohydrate, the name amyloid has been retained. In 1922, Bennhold introduced the use of the dye Congo red to stain tissue amyloid deposits. After the dye binds, it exhibits an apple-green birefringence when examined by polarized microscopy that sharply distinguishes amyloid from other tissue components (Figure 1). Ever since, Congo red has been the primary method by which pathologists identify amyloid deposits in tissue samples. In 1927, Divry showed that the cores of senile plaques, the cortical structures that Alzheimer linked with early-onset senile dementia in his classic 1906 paper, exhibit the Congo red birefringence characteristic of amyloid. Amyloid plaques are recognized as one of the pathological hallmarks of Alzheimer's disease (AD) and it is believed that some aggregated form of the main protein component of the amyloid fibrils in these plaques plays a direct role in AD etiology. Although it had long been recognized that the main chemical component of amyloid fibrils is protein, it was not until the pioneering work of the Glenner and Benditt groups in the early 1970s that it was realized that amyloid fibrils are predominantly composed of *specific* protein sequences rather than a broad mixture of protein molecules. Glenner showed that amyloid extracted from primary amyloidosis patients contained the immunoglobulin light chain molecule, and Benditt found that amyloid from tissues of chronic inflammation patients consisted of a new protein called amyloid A. Other clinically distinct forms of amyloid

 © 2004, Elsevier Inc. All Rights Reserved. 

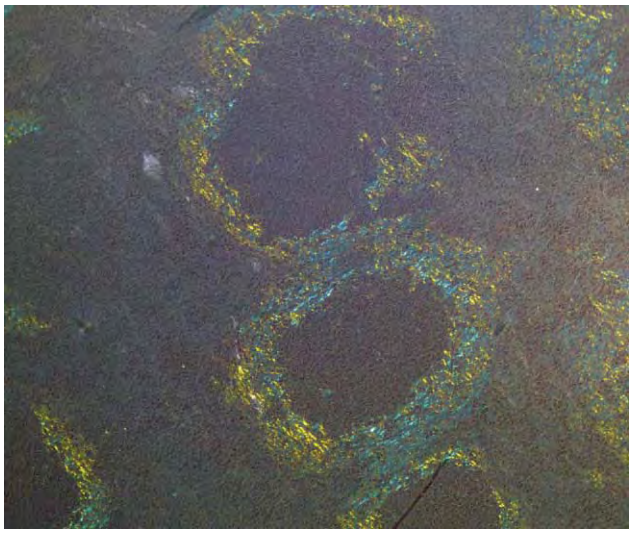


**FIGURE 1** Rings of amyloid detected by Congo red staining and polarized microscopy in the spleen of a mouse with serum amyloid A amyloidosis. Courtesy of Professor Jonathan Wall, University of Tennessee.

were soon thereafter shown to have different characteristic protein constituents. In the mid-1980s, Glenner isolated and characterized by amino acid sequencing the previously unknown peptide A$\beta$ as the principal component of AD amyloid fibrils. By the end of the 20th century, many naturally occurring amyloid proteins had been characterized and the list continues to grow with the development of more sensitive means of analysis.

## Amyloid Diseases

### Range of Amyloid-Related Diseases

Table I lists some of the over 30 human disease states in which amyloid fibrils have been observed, as characterized by the principal organ involved and the principal protein component of each. Amyloid diseases can occur both in the brain and in the rest of the body. The systemic amyloid diseases tend, as a general rule, to involve large deposits of amyloid that in extreme cases dramatically increase the size of the affected organ. In these cases, the toxicity of the amyloid deposit may be largely due to the mass of deposited material and its ability to disrupt normal tissue structure and function. In some peripheral amyloid diseases, the amyloid deposits are localized to a particular tissue. Such is the case in pancreatic amyloid, which is found in over 90% of adult-onset (type 2) diabetes patients (despite this high incidence, the pathogenic role of amyloid deposition in diabetes is not yet known). In contrast, in other amyloidoses, protein deposits can be found in a variety of tissues. Amyloid is also observed in a number of brain diseases. In AD, extracellular amyloid deposits are found in the cerebral cortex, usually embedded with fragments of neuronal processes and surrounded by activated glial cells. In Huntington's disease, amyloid deposits are found in both the cytoplasm and the nucleoplasm of particular neurons in the cortex and elsewhere. In Parkinson's disease, large neuronal deposits called Lewy bodies are rich in the protein $\alpha$-synuclein. In each of these brain diseases, the mechanistic role of protein deposits has not yet been established.

**TABLE I**

**Major Polypeptide Components of Pathogenic Amyloid Deposits in Humans**

| Polypeptide | Major disease states |
|---|---|
| Transthyretin | Heart, kidney, peripheral neuropathy |
| Serum amyloid A | Kidney, peripheral neuropathy |
| Immunoglobulin light chain | Kidney, heart |
| Immunoglobulin heavy chain | Spleen |
| $\beta_2$-Microglobulin | Carpal tunnel syndrome, osteoarthropathies |
| Lysozyme | Nonnaturopathic visceral amyloid |
| Islet amyloid polypeptide | Diabetic pancreatic islet cells |
| Fibrinogen $\alpha$-chain | Kidney |
| Apolipoprotein A1 | Peripheral neuropathy, liver |
| Atrial natriuretic peptide | Heart |
| Amyloid $\beta$-protein (A$\beta$) | Brain (Alzheimer's disease, cerebral amyloid angiopathy) |
| $\alpha$-Synuclein | Brain (Parkinson's disease) |
| *huntingtin* polyglutamine sequence | Brain (Huntington's disease) |
| Prion protein (PrP) | Brain (Creutzfeldt–Jakob disease, mad cow disease) |
| Cystatin C | Brain (cerebral amyloid angiopathy) |
| Gelsolin | Brain (cerebral amyloid angiopathy) |
| ABri | Brain (familial British dementia) |

### Physiological Factors in Amyloid Formation

Although much remains to be learned about the events that trigger amyloid deposition, it is clear that a variety of factors are involved. Some well-established factors are molecular aspects of the proteins themselves. Since the rates of aggregation reactions, such as amyloid formation, depend on concentration, dramatic increases in the amount of the "amyloidogenic" precursor protein can initiate amyloid formation. This is the case for the protein serum amyloid A, which increases in concentration in response to infection and, when persistently elevated, can deposit as amyloid. Similarly, the reason Down's syndrome patients invariably develop AD may be because the extra chromosome associated with the

disease contains the gene for the amyloid $\beta$-protein precursor (APP), the protein from which the main plaque component A$\beta$ is generated. The presence of a third active copy of the APP gene in cells results in increased levels of A$\beta$, which in turn serves to initiate, or "nucleate," fibril formation. One of the unsolved mysteries of amyloid disease is how nucleation of amyloid growth occurs in most disease states. When studied *in vitro*, proteins responsible for amyloid disease often require extremely high, nonphysiological concentrations for nucleation and growth of amyloid fibrils. This indicates that there are unknown specific structures and/or environments in the body that facilitate nucleation. Amyloid plaques typically contain other proteins in addition to the main fibril component and one role of some of these proteins may be to contribute to amyloid formation by helping to stabilize the fibrils and protect them from normal degradative mechanisms. Amyloid diseases are often age-related, leading to the speculation that sporadic amyloid deposition may be a not uncommon occurrence that is held in check in the young and healthy but which advances when normal surveillance mechanisms break down in the aged. This may account for the finding that, in the inherited amyloidosis associated with mutant forms of amyloidogenic precursor proteins, the diseases generally occur later in life, despite the fact that the proteins are expressed from birth.

## Prions and Amyloid Enhancement Factor

In one group of amyloid-related brain diseases, a group including Creutzfeldt–Jakob disease, mad cow disease, and the sheep disease scrapie, the conditions can be transmitted by ingestion of "prion" particles, misfolded versions of a normal cellular protein, from the nervous system tissue of a previous victim. Although it is not firmly established that the actual prion particle or state is itself an amyloid fibril, the ability of prion infectivity levels to be amplified in the body bears an intriguing resemblance to the ability of amyloid fibrils to amplify themselves from a pool of precursor protein, through seeding. All mammals express a version of the prion precursor protein, PrP, which has a normal function and does not normally lead to disease. Except in rare instances of sporadic and genetic forms of the disease, prion-associated diseases are observed only when the animal has been exposed to exogenously supplied prion particles, which appear to act *in vivo* as seeds or templates for the propagation of new prions from the normal PrP pool. This scenario is reminiscent of the phenomenon that serum amyloid A amyloidosis can be accelerated in experimental animals by administration of an agent called amyloid enhancement factor (AEF). AEF extracted from the amyloidotic spleen of a mouse and injected intravenously into new mice can induce the rapid development of amyloid deposits compared to untreated controls. Biochemical studies are consistent with AEF being essentially composed of amyloid fibrils, although the presence of an important minor component is difficult to rule out. A possible strong connection between the AEF and prion phenomena has been suggested by experiments showing that AEF, as well as pure amyloid fibrils created *in vitro*, can induce amyloid disease in mice when placed in their drinking water. Thus, although most amyloid diseases are not considered to be transmissible, the results in this experimental system suggest that various amyloid fibrils might be capable of behaving as prions in some circumstances.

## Toxicity of Amyloid Fibrils

Although in some peripheral conditions, amyloid can cause life-threatening disease by accumulating in such high mass that normal tissue structure and function are disrupted, in other cases, particularly in the neurodegenerative diseases, the accumulated mass of amyloid is very low compared to the surrounding cell mass. In these cases, the mechanism(s) by which amyloid fibrils cause cell death or cell dystrophy is not at all clear. Whether there is a uniform toxic mechanism in all diseases or different mechanisms for different diseases is also unknown. Toxicity mechanisms under investigation include the following: (1) collateral damage caused by immune responses to an amyloid deposit; (2) membrane depolarization resulting from channels created by amyloid fibril assembly intermediates inserted into membranes; (3) recruitment of other proteins into growing aggregates, which has the effect of denying the cell the activity of the recruited protein(s); (4) disruption or overwhelming of the normal cellular apparatus for breakdown and elimination of misfolded proteins, such as the ubiquitin – proteasome system and the molecular chaperones.

## Evolved Amyloid

As far as is known, most protein sequences in evolution were selected for their abilities to efficiently access functional, folded states, while being either unselected, or selected against, with respect to the ability to make amyloid fibrils. Thus, in most cases where amyloid forms, the amyloid can be viewed as an accidental, environmentally induced structure never intended by nature. There are several examples, however, of proteins whose ability to make amyloid fibrils in microbial cells is beneficial to the organism. Yeast prions, for example, function through their ability to form and seed amyloid fibrils and in doing so they modulate the levels of the soluble form of the prion protein in the cell, the fluctuations in which play metabolic and ultimately regulatory roles. In another example, a complex system

of proteins in *Escherichia coli* is responsible for producing an extracellular amyloid fibril that plays a role in cell adhesion. The existence of functional, beneficial amyloid might be viewed as an example of nature's ability to exploit to advantage the novel properties of the products of evolution.

## Amyloid Proteins

### Range of Amyloid Proteins

Amyloid fibrils composed of different proteins share a number of common structural features, despite the fact that these proteins, in their native states, vary widely in structure, cellular locations, and properties. For example, the amyloid protein transthyretin is a tetramer of subunits with a total molecular weight of 55 kDa. In contrast, small peptides of approximately 40 amino acid residues, such as A$\beta$ and islet amyloid polypeptide (IAPP), and fragments of IAPP as short as pentapeptides, are also capable of amyloid fibril formation. Proteins can grow into amyloid fibrils regardless of the nature of their normal folding patterns. In their native states, $\alpha$-synuclein exhibits no stable structure in solution, transthyretin is dominated by $\beta$-sheet, lysozyme is a mixed $\alpha$-helix/$\beta$-sheet protein, and serum amyloid A is essentially fully $\alpha$-helical, yet all are capable of forming amyloid fibrils. Most of the peripheral amyloidoses involve secreted proteins, whereas the nine different proteins responsible for nine expanded polyglutamine diseases – including Huntington's disease – are cellular proteins (which, in turn, are found in a variety of subcellular locations). Most amyloid proteins contain significant percentages of hydrophobic residues, but polyglutamine consists entirely of the relatively polar amino acid glutamine. One of the few patterns shared among amyloidogenic protein regions is the infrequent occurrence of proline residues, which strongly disfavor $\beta$-sheet – the dominant secondary structural feature of the amyloid fibril.

### Molecular Factors in Amyloid Formation

Until the early 1990s, the unusually insoluble, fibrous, quasi-crystalline amyloid fibril was generally viewed as a structural form in which the subunit building block was essentially the native state of the amyloidogenic precursor protein, in a model analogous to structural models for aggregated sickle cell hemoglobin. A significant advance in the understanding of amyloidosis was the demonstration in the mid-1990s that amyloid formation is strongly enhanced when the folded state of a protein is destabilized by mutation or by the solution environment, indicating that fibril structure involves nonnative states. These studies were later extended to show that denaturing conditions can induce amyloid formation even in some proteins not known to be pathogenic. It is clear that misfolding, i.e., the rearrangement of the complex folded shape of a protein molecule, is central to amyloid formation. Thus, protein stability – the resistance of the folded conformation to misfolding/unfolding – is an important factor in determining susceptibility to amyloid formation. Extreme environments in the body, such as acidic cell compartments, may, in some cases, facilitate protein unfolding and therefore promote amyloid formation. Proteolytic removal of a portion of a protein by an endogenous protease can also be a destabilizing factor leading to amyloid formation. Notably, mutations that alter the primary structure of proteins can affect protein stability. In fact, many of the amyloid diseases involve amino acid substitutions in an amyloid precursor protein. The most common example is familial amyloidosis, caused by a variety of single point mutations in the protein transthyretin. In addition, noninheritable, specific amino acid changes in immunoglobulin light chains are linked to a high risk of amyloidosis. Amino acid changes can contribute to the efficiency of amyloid formation not only by modifying the stability of the native state of the precursor protein, but also by modifying the stability of the amyloid fibril itself; an example of the latter is the ability of proline residues to destabilize amyloid structure.

### Amyloid Assembly

The manner in which amyloid fibrils grow can be considered in terms of the kinetics of the process or in terms of the structures of assembly intermediates. Kinetically, amyloid fibril formation *in vitro* typically exhibits a lag time of hours to days, followed by a rapid growth phase. In many such cases, the lag phase can be abbreviated or eliminated if a small amount of fibril is provided as seed at the start of the reaction. Such behavior has been interpreted as evidence that fibril growth takes place by nucleated growth polymerization, a mechanism of colloidal assembly in which the initiation of aggregate growth depends on the sporadic generation of a highly unstable, highly organized nucleus state. However, it is far from clear how amyloid growth is initiated *in vivo* and it is possible, even likely, that other molecules or structures are involved, which would make the *in vivo* mechanism more akin to what in physics is referred to as heterogeneous nucleation. *In vitro*, after the nucleation (or seeding) phase has occurred, growth continues predominantly via the elongation of existing fibrils and it has been argued that this is a more realistic view of how amyloid develops *in vivo*. A number of assembly intermediates have been detected when amyloid growth is studied *in vitro*. Whether or not any of these can be considered

to be true kinetic nuclei, some of these structures may have relevance *in vivo*. Amyloid growth from monomeric proteins seems to proceed via the formation of successively larger, more complex structures, from small globular aggregates to short curvilinear filaments to isolated straight and unbranched fibrils to bundles of fibrils. The early assembly intermediates in this progression appear to be more cytotoxic than the mature fibrils, leading to the speculation that assembly intermediates are the real culprits in disease pathogenesis. Little is known, however, about whether and how these intermediates might be formed in the body and how they might kill cells.

## Amyloid Structure

Amyloid ultrastructure as revealed by electron microscopy (Figure 2) or atomic force microscopy shows fibrils to be relatively straight and unbranched, with diameters in the range of 80–160Å. Fibrils have a characteristic twist, being composed of two to six protofilaments of diameter 30–40Å. X-ray fiber diffraction studies reveal that fibrils and their constituent protofilaments are rich in a type of $\beta$-sheet structure known as cross-$\beta$. In this conformational structure type, the extended chain elements of the $\beta$-sheet are perpendicular to the fibril axis and the hydrogen bonds between the strands are parallel to the fibril axis. Beyond this level of resolution, little is known about the detailed protein folding of the amyloid motif because of imposing technical barriers to their study by the standard methods of X-ray crystallography and solution-phase nuclear magnetic resonance spectroscopy. Nevertheless, a number of structural models have been proposed and important insights continue to be obtained through lower resolution studies. The structure of the amyloid folding motif is important for a number of reasons, only one of which is their association with a variety of human diseases. It is clear that misfolded, aggregated proteins are formed routinely in the cell due to fundamental inefficiencies in the protein folding process; understanding amyloid structure may help elucidate how the cell manages to recognize and eliminate the products of folding mishaps. Mapping amyloid structure and assembly will also contribute to understanding of this previously ignored aspect of the protein folding reaction – unproductive side products. Finally, better structural information may allow a rational design approach to the development of anti-amyloid therapeutics.

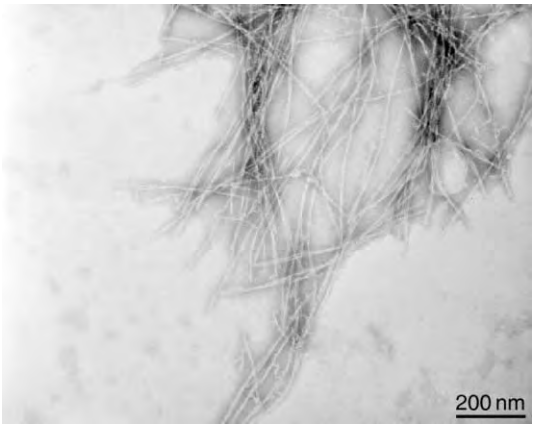



**FIGURE 2** An electron micrograph of an amyloid fibril grown in the laboratory from the Alzheimer's disease peptide A$\beta$. Courtesy of Professors Indu Kheterpal and John Dunlap, University of Tennessee.

## See Also the Following Articles

Cell Death by Apoptosis and Necrosis • Chaperones, Molecular • Prions and Epigenetic Inheritance • Prions, Overview • Proteasomes, Overview • Ubiquitin System

## Glossary

**folding motif** The three-dimensional shape pattern exhibited by a folded protein, consisting of the secondary structural units (e.g., $\alpha$-helix, $\beta$-strands) accessed by different elements of the primary sequence, and how those secondary structural units interact with one another in space. Although there are tens of thousands of protein sequences coded within the human genome, it is believed that the three-dimensional structures of these proteins will ultimately be described by perhaps only a few hundred folding motifs.

**glial cells** Nonneuronal, accessory cells in the brain that are responsible for maintaining brain structure and development and for fighting infection.

**membrane depolarization** Loss of chemical potential across a membrane.

**molecular chaperones** Enzymatic protein assemblies that are responsible for detecting, reversing, and, where necessary, eliminating failed products of protein folding, such as misfolded and aggregated proteins.

**nuclear magnetic resonance** A type of spectroscopy in which the unique covalent and noncovalent environments of atoms in molecules in solution can be assessed, allowing for construction of a high-resolution structural model of the three-dimensional relationships between atoms.

**ubiquitin–proteasome system** A complex pathway of enzymes responsible for marking and destroying proteins that are no longer required by the cell due to their being obsolete or defective. Such proteins are first marked by the attachment of the small protein ubiquitin, at which point they are recognized and destroyed by the proteasome, a large protein machine consisting of proteases and other enzyme activities.

**X-ray crystallography** A technique for determining at high resolution the spatial relationships between atoms in a molecule in the solid state, by the detailed diffraction pattern generated when a molecular crystal is exposed to X-ray beams.

**X-ray fiber diffraction** An intermediate-resolution version of X-ray diffraction analysis, not requiring a crystalline form, in which repeated patterns of a structure can be recognized from a limited diffraction pattern.

## Further Reading

Dobson, C. M. (2001). The structural basis of protein folding and its links with human disease. *Philos. Trans. R. Soc. London B Biol. Sci.* **356**, 133–145.

Falk, R. H., Comenzo, R. L., and Skinner, M. (1997). The systemic amyloidoses. *N. Engl. J. Med.* **337**, 898–909.

Kelly, J. W. (1998). The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. *Curr. Opin. Struct. Biol.* **8**, 101–106.

Lindquist, S. (1997). Mad cows meet psi-chotic yeast: The expansion of the prion hypothesis. *Cell* **89**, 495–498.

Martin, J. B. (1999). Molecular basis of the neurodegenerative disorders. *N. Engl. J. Med.* **340**, 1970–1980. [Published erratum appears in *N. Engl. J. Med.* (1999). **341**, 1407].

Selkoe, D. J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* **399**, A23–A31.

Sipe, J. D., and Cohen, A. S. (2000). Review: History of the amyloid fibril. *J. Struct. Biol.* **130**, 88–98.

Wetzel, R. (1994). Mutations and off-pathway aggregation. *Trends Biotechnol.* **12**, 193–198.

## Biography

Dr. Ronald Wetzel is a Professor of Medicine in the Graduate School of Medicine at the University of Tennessee in Knoxville. He obtained a Ph.D. in physical organic chemistry from the University of California, Berkeley, and completed postdoctoral studies at the Max–Planck Institute for Experimental Medicine and at Yale University. He participated in the development of the modern biotechnology industry as an early employee at Genentech, Inc. Dr. Wetzel's major research focus is in the structures and assembly mechanisms of misfolded proteins and protein aggregates, in how they contribute to human disease, and in developing therapeutic interventions for these diseases.

# Anaplerosis

**Raymond R. Russell, III**
*Yale University School of Medicine, New Haven, Connecticut, USA*

**Heinrich Taegtmeyer**
*University of Texas-Houston Medical School, Houston, Texas, USA*

**The word anaplerosis (from the Greek, meaning "to fill up") was coined in the 1960s by Sir Hans Kornberg to describe metabolic processes that replenish intermediates in a biochemical cycle. Anaplerotic pathways are present in both eukaryotic and prokaryotic organisms. This article discusses anaplerosis as it relates to the citric acid cycle in mammalian tissues with reference to organ function. Metabolic cycles are essential for the efficient transfer of energy in the cell. In organs such as the heart and skeletal muscle, a series of moiety-conserved cycles connects the circulation with the cycling of crossbridges in the contractile elements of sarcomeres. Here we focus on anaplerotic mechanisms that maintain the intermediates of one of the most important metabolic cycles.**

## Introduction

Steady-state concentrations of intermediates in a metabolic pathway depend on their rates of synthesis and degradation. Substrates entering the citric acid cycle as citrate (via the condensation of acetyl-CoA with oxaloacetate) do not cause a net change in the citric acid cycle pool size. The two carbons of acetyl-CoA are lost as $CO_2$ in the isocitrate and the $\alpha$-ketoglutarate dehydrogenase reactions. In contrast, anaplerosis supplies compounds to the citric acid cycle through reactions other than those catalyzed by citrate synthase. These compounds replenish carbon intermediates lost primarily as amino acids (glutamate or aspartate) or as oxaloacetate. The role of anaplerosis in the citric acid cycle is twofold. First, as intermediates are drained away during synthetic processes such as gluconeogenesis, glyceroneogenesis, and amino acid synthesis, anaplerosis replaces them, allowing citric acid cycle flux to proceed without impairment. This functional aspect of anaplerosis is especially important in the liver and renal cortex. Second, anaplerosis facilitates energy production in organs with high rates of energy turnover, such as the heart and skeletal muscle. The citric acid cycle operates in two spans, and flux is regulated by the $\alpha$-ketoglutarate dehydrogenase reaction. Under normoxic conditions, faster energy turnover leads to an increase in the pool size of citric acid cycle intermediates. However, under hypoxic conditions, flux through a portion of the citric acid cycle provides anaerobic energy by substrate level phosphorylation.

## Anaplerotic Pathways

### Substrates for Anaplerosis

As a key component of all living cells, the citric acid cycle is highly organized and must be able to respond to constant changes in its environment. It is thought to be tied into a distributive system of energy transformation; however, this distributive control theory is difficult to prove experimentally, even though regulation of anaplerotic flux through a variety of pathways is part of this response (Figure 1).

The primary substrates for anaplerosis are pyruvate and alanine, although glutamate can be a physiologically significant substrate as well. In addition, the branched-chain amino acids valine and isoleucine, as well as odd-chain fatty acids and their degradation product, propionate, serve as anaplerotic substrates.

### Entry at Malate and Oxaloacetate

A major anaplerotic substrate in the heart is pyruvate. Although most pyruvate is oxidatively decarboxylated and enters the citric acid cycle as acetyl-CoA, a portion is also carboxylated and enters the C4 pool of the citric acid cycle. This reaction is catalyzed by either pyruvate carboxylase (forming oxaloacetate) or by NADP-dependent malic enzyme (forming malate). Even though the NADP-dependent malic enzyme reaction is reversible, the reaction in the direction of malate production is slow and unlikely to play a significant role in anaplerosis. Aspartate can also enter the citric acid cycle as a C4 intermediate. It is transaminated with $\alpha$-ketoglutarate via aspartic aminotransferase to form oxaloacetate and

 © 2004, Elsevier Inc. All Rights Reserved. 

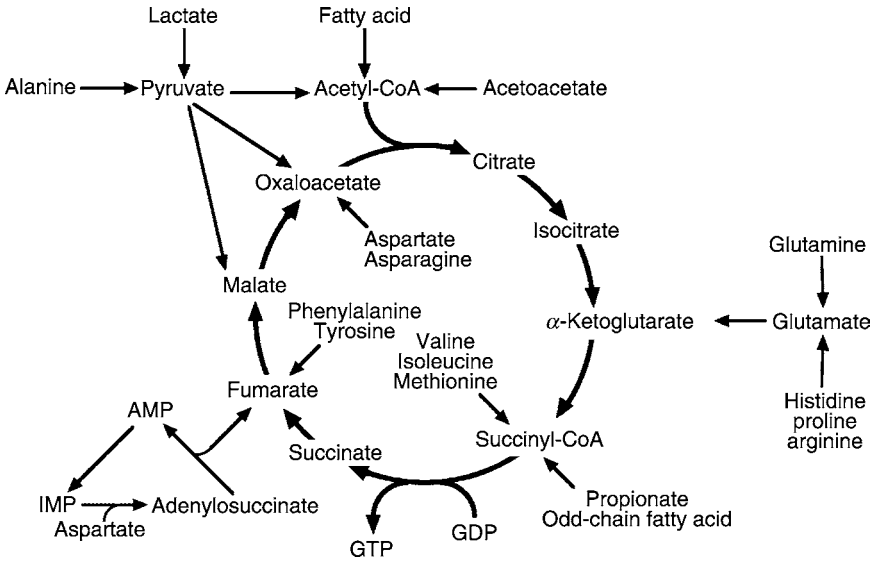



**FIGURE 1** Summary of anaplerotic pathways leading to the citric acid cycle.

glutamate. In this reaction, anaplerosis balances the loss of $\alpha$-ketoglutarate; however, the exact stoichiometry of these reactions has not yet been determined.

### Entry via $\alpha$-Ketoglutarate

In heart muscle (which has the highest rate of oxygen consumption of all mammalian organs), $\alpha$-ketoglutarate dehydrogenase activity correlates with the rate of oxygen consumption. Therefore, it is likely that $\alpha$-ketoglutarate dehydrogenase is rate limiting for citric acid cycle flux. Indeed, isotopomer analysis of glutamate enrichment from acetyl-CoA is a lap counter for measuring citric acid cycle flux. A fundamental assumption is that $\alpha$-ketoglutarate leaves the cycle as glutamate. This reaction is readily reversible by mass action when $\alpha$-ketoglutarate is formed from glutamate via transamination with pyruvate (forming alanine) or oxaloacetate (forming aspartate). Furthermore, in skeletal muscle glutamate is converted to $\alpha$-ketoglutarate and $NH_4^+$ by glutamate dehydrogenase. This enzyme shows no activity in heart muscle.

### Entry via Succinyl-CoA

The citric acid cycle intermediate succinyl-CoA plays an important role in fatty acid and amino acid metabolism because it is the entry point of odd-chain fatty acids, propionate, and the branched-chain amino acids valine and isoleucine into the citric acid cycle. Substrate entry as $\alpha$-ketoglutarate or succinyl-CoA, in contrast to other anaplerotic pathways, is associated with the generation of high-energy phosphates. The reaction catalyzed by succinyl-CoA synthetase is reversed and leads to substrate-level phosphorylation of GDP to GTP. This energy-producing pathway becomes important in myocardial ischemia when ATP generation by oxidative phosphorylation is inhibited.

### Entry via Fumarate

In addition to the amino acids phenylalanine and tyrosine entering as fumarate, the purine nucleotide cycle enriches the citric acid cycle based on the net reaction:

$$\text{aspartate} + \text{GTP} \rightarrow \text{fumarate} + NH_3 + \text{GDP} + P_i$$

Previous work has demonstrated that flux through the purine nucleotide cycle increases in skeletal muscle during intense exercise. This increased flux has two effects: First, it can maintain the pool of adenine nucleotides, and second, it can increase the citric acid cycle pool size via anaplerosis. Both of these effects are expected to improve energy metabolism in exercising muscle.

## Exit of Intermediates: Balancing Anaplerosis

By definition, under steady-state conditions, substrates entering the citric acid cycle via anaplerotic pathways are balanced by removing an equivalent amount of citric acid cycle intermediates via pathways that maintain a constant citric acid cycle pool size. These pathways have been termed cataplerotic, although the term is a misnomer; instead, the word drainage seems more appropriate. Drainage pathways generally involve removing the citric acid cycle intermediates oxaloacetate

(as aspartate via transamination or as phosphoenolpyruvate via decarboxylation by phosphoenolpyruvate carboxykinase), citrate, or α-ketoglutarate (via transamination to glutamate). Although these drainage pathways are usually thought of as means to balance anaplerotic pathways, they play critical roles in renal gluconeogenesis and enterocyte energy production from glutamine (see later discussion).

## Measuring Anaplerosis

### Changes in Citric Acid Cycle Pool Size

There are two principal methods of assessing anaplerosis based on changes in citric acid cycle pool size. The amount of citric acid cycle pool intermediates can be measured enzymatically or resolved with high-performance liquid chromatography. Although both methods can reliably assess changes in the citric acid pool size, they do not provide an insight into the pathways involved in anaplerosis nor do they determine relative rates of enrichment. Furthermore, because anaplerosis is usually balanced by the exit of intermediates, changes in citric acid pool size are generally negligible.

Simply determining changes in citric acid cycle pool size provides no information on the rates of anaplerosis or on specific anaplerotic reactions. Using substrates labeled with tracer $^{14}C$ and measuring incorporation into specific positions in citric acid cycle intermediates is more revealing. This method can not only determine rates of anaplerosis, but also characterize the pathways involved in the process. However, the method is laborious and prone to error. It requires the sequential enzymatic degradation of intermediates and quantitation of the release of the $^{14}C$ label. It has been replaced by $^{13}C$-nuclear magnetic resonance (NMR) spectroscopy or mass spectroscopy.

### $^{13}C$-Nuclear Magnetic Resonance-Mass Spectroscopy

$^{13}C$-NMR spectroscopy is used to assess quantitatively the relative contributions of various substrates to the citric acid cycle. This method determines the anaplerotic enrichment of citric acid cycle intermediates such as the radiotracer method already discussed, but without digesting the carbon skeleton. $^{13}C$-labeled compounds enter the carbon skeleton of citric acid cycle intermediates at specific positions through anaplerotic pathways or through the dilution of $^{13}C$ by the entry of unlabeled anaplerotic substrates. The enrichment of $^{13}C$ at those positions is then assessed, indicating citric acid cycle intermediate enrichment. The method is illustrated in Figure 2, in which the enrichment of the carbon skeleton

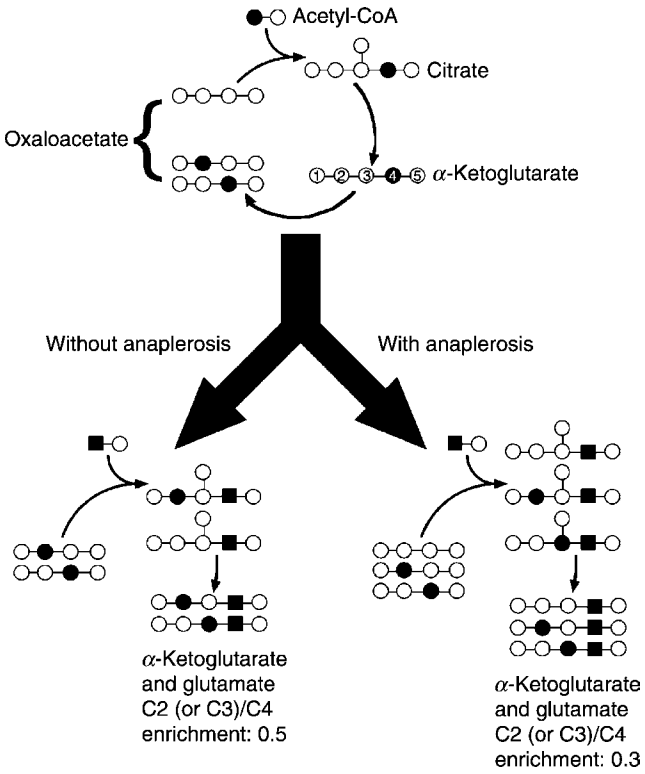



**FIGURE 2** Fundamental concept of assessing anaplerotic flux using $^{13}C$-NMR spectroscopy as described by Malloy *et al.* (1988). The black circles represent $^{13}C$ that enters the citric acid cycle pool via the first turn of the citric acid cycle. The black squares represent $^{13}C$ that enters the citric acid cycle pool via the second turn of the citric acid cycle.

of α-ketoglutarate (and glutamate) at the C2, C3, and C4 positions is used to quantify anaplerosis. Investigators have used the following strategy. Using [2-$^{13}C$]acetyl-CoA as a substrate and conditions in which there is no anaplerotic flux, one can assume that there is no dilution of the $^{13}C$ label in the C2 or C3 position of α-ketoglutarate. The label in these positions is generated from the randomization of the C4-position carbon in the first turn of the citric acid cycle. Therefore, the sum of the $^{13}C$ enrichments of the C2 and C3 carbons will be equal to the enrichment of the C4 carbon of glutamate. In contrast, with anaplerotic activity, the labeled C2 and C3 carbons will be diluted by $^{12}C$ arising from anaplerotic substrates, and the sum of the enrichments of the labeled C2 and C3 carbons will be less than the enrichment of the C4 carbon of glutamate.

### Expression and Activity of Proteins Regulating Anaplerosis

Using the methods outlined, studies have focused on changes in enrichment of citric acid cycle intermediates to assess the activity of anaplerotic pathways and

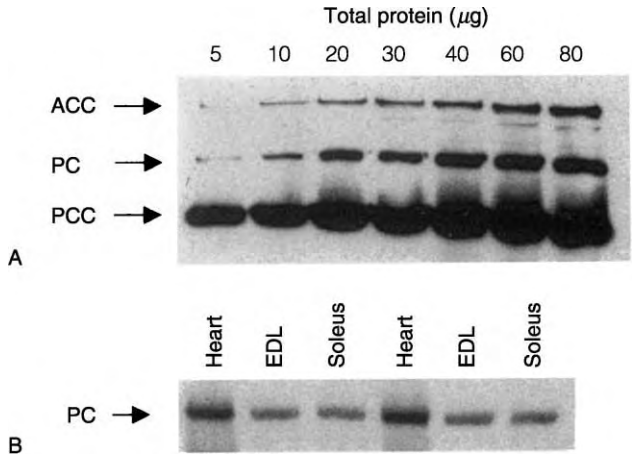



**FIGURE 3** Expression of the biotin-containing carboxylases, acetyl-CoA (ACC), pyruvate carboxylase (PC), and propionyl-CoA carboxylase (PCC) in heart muscle based on (A) streptavidin blotting and (B) pyruvate carboxylase expression in rat heart and skeletal muscle. Reprinted from Gibala *et al.* (2000), *Acta Physiol. Scand.* **168**, 657–665, Scandinavian Physiological Society.

explain changes in flux in terms of mass action and allosteric regulation of key enzymes. Although this is most likely the case with acute changes in anaplerotic flux, changes in the expression and activity of enzymes responsible for anaplerosis should be taken into account when studying chronic processes such as pressure-overload-induced hypertrophy, heart failure, or diabetes. In streptozocin-treated diabetic rats, transcript levels of enzymes in anaplerosis in heart and skeletal muscle are down-regulated (Figure 3). Changes in transcript levels are paralleled by similar changes in protein expression. Although, the *in vitro* activities of the anaplerotic enzymes involved in the carboxylation of substrates (e.g., pyruvate carboxylase) can be measured by determining the incorporation of $^{14}C$ from $H^{14}CO_3^{-1}$, it is not known whether changes in expression correlate with changes in activity.

## Changes in Anaplerosis in Response to Environmental Stress: Workload, Nutritional Status, and Disease

Ultimately, any metabolic process has functional consequences, and anaplerotic pathways are no exception. Anaplerotic pathways play important roles in regulating a wide variety of organ responses to conditions of metabolic stress ranging from exercise to inborn errors of metabolism.

### Anaplerosis in Skeletal Muscle during Exercise

The transition from rest to moderate or intense exercise is associated with large increases in skeletal muscle ATP turnover, implying increases in citric acid cycle flux (up to 100-fold increases). Although, citric acid cycle intermediates increase only three- to fourfold, these relatively small increases in the citric acid cycle pool size (via anaplerosis) can reflect dramatic increases in citric acid cycle flux. It has been suggested that the increases allow skeletal muscle to adapt to the energetic demands of exercise, but, interestingly, there is no appreciable change in citric acid cycle pool size at lower levels of exercise. The majority of changes in individual intermediates occur in the second span of the citric acid cycle (i.e., from succinate to oxaloacetate) because the primary source of enrichment during acute exercise appears to be flux through the reaction catalyzed by alanine aminotransferase. This reaction results in entry of glutamate as $\alpha$-ketoglutarate; however, pyruvate carboxylase and malic enzyme may contribute minor amounts to citric acid cycle enrichment in exercising muscle.

### Glutamine Metabolism by the Small Intestine

Glutamine is a source of energy for a number of specialized tissues, including the small intestine. When glutamine is taken up by the small intestine, it enters the citric acid cycle as $\alpha$-ketoglutarate and leaves the cycle through oxidation as $CO_2$. More specifically, $\alpha$-ketoglutarate is converted to malate by citric acid cycle reactions and malate may be transported out of the mitochondria. In the cytosol, malate is converted to oxaloacetate. As in the liver and heart, the malate-aspartate shuttle is bidirectional. It can transport electrons from extramitochondrial NADH into the mitochondria or from intramitochondrial NADH to the cytosol.

### Renal Ammonia Formation during Starvation

During starvation, when protein breakdown, renal gluconeogenesis, and hepatic ketogenesis increase, the rate of renal ammoniagenesis also increases. Ammonia is generated from amino acids, including glutamine, released by skeletal muscle. Glutamine is converted to $\alpha$-ketoglutarate in renal cells; $\alpha$-ketoglutarate is metabolized to malate; and malate is transported out of the mitochondria, oxidized to oxaloacetate and to phosphoenolpyruvate, and ultimately used for gluconeogenesis. Although different from glutamine oxidation by

enterocytes, this series of reactions also reflects the balance that is generally observed between anaplerotic and drainage pathways.

## Ischemia and Substrate-Level Phosphorylation

Early studies assessing cardiac metabolism by measuring arteriovenous differences in amino acid concentrations revealed that, in patients with coronary artery disease and myocardial ischemia, the heart avidly takes up glutamate and releases alanine. This finding led to the hypothesis that the anaplerotic substrate glutamate enters the citric acid cycle as $\alpha$-ketoglutarate via transamination with pyruvate (thereby forming the alanine that is released). Subsequently, the $\alpha$-ketoglutarate is metabolized to succinate with the concomitant substrate-level phosphorylation of GDP to form GTP (Figure 4A). In this way, a span of the citric acid cycle can generate high-energy phosphates in the absence of sufficient oxygen for full citric acid cycle operation. Translational research based on this anaplerotic pathway has led to the development of glutamate-enriched solutions that increase anaerobic energy production of the heart during coronary artery bypass surgery.

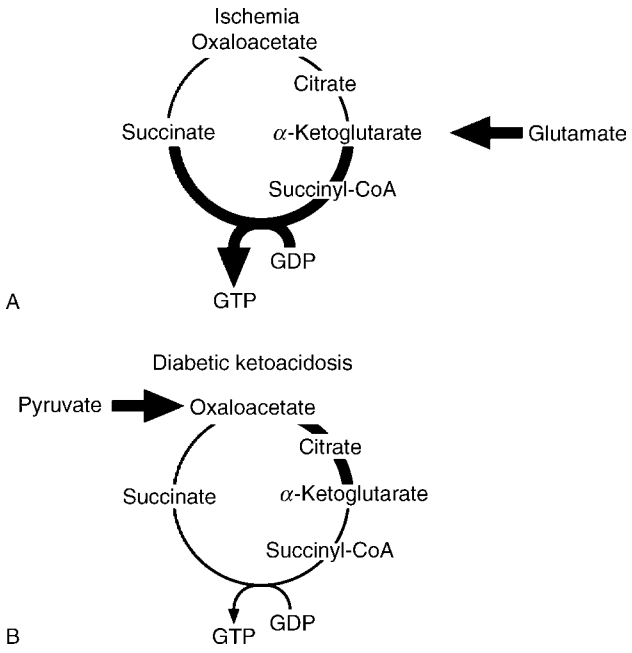



**FIGURE 4** Role of anaplerosis in improving myocardial energetics in the setting of (A) ischemia and (B) diabetic ketoacidosis. Under both conditions, loss of cofactors ($NAD^+/FAD^+$ for ischemia, CoASH for diabetic ketoacidosis) inhibits full citric acid cycle activity. Anaplerotic pathways allow the citric acid cycle to work in spans, thereby increasing the production of high-energy phosphates. Adapted from Taegtmeyer and Passmore (1985).

## Diabetes and Ketone Body Metabolism

Citric acid cycle pool size increases in the hearts of rats with experimentally induced diabetes, suggesting enrichment by anaplerotic pathways. We have suggested that an increase in anaplerotic flux, which primarily occurs through pyruvate carboxylation (via malic enzyme), plays an important role in maintaining flux through the second span of the citric acid cycle. Acutely, the metabolic derangement of ketoacidosis that occurs with diabetes inhibits flux through $\alpha$-ketoglutarate dehydrogenase by sequestration of coenzyme A (CoASH). This phenomenon is associated with contractile dysfunction of the heart that can be readily reversed by the addition of glucose, lactate, or pyruvate (all of which are anaplerotic substrates). The effects of pyruvate are mediated by enrichment of malate in the citric acid cycle pool, which occurs by carboxylation of pyruvate to form malate and oxaloacetate through the actions of malic enzyme and pyruvate carboxylase, respectively (Figure 4B). The citric acid cycle is thereby able to operate once again in a span that can generate reducing equivalents to support oxidative phosphorylation of ADP to form the ATP necessary to drive the contractile machinery of the heart.

## Long-Chain Fatty Acid Oxidation Defects and Myopathies

The inability to oxidize long-chain fatty acids due to deficiencies in activity of carnitine palmitoyltransferase-1 or the enzymes involved in $\beta$-oxidation is associated with contractile dysfunction due to skeletal and heart muscle damage. One proposed mechanism for the decrease in contractile activity is decreased citric acid cycle flux due to loss of intermediates from damaged myocytes. Based on this hypothesis, a recent trial treated patients with defects in long-chain fatty acid oxidation with odd-chain triglycerides (which can increase the citric acid cycle pool size by entering as succinyl-CoA) and tested the hypothesis that an increase in citric acid cycle pool size may improve muscle function. The study demonstrated beneficial effects, such as reversing left-ventricular dysfunction, decreasing muscle breakdown, and decreasing weakness.

# Summary and Perspective

Efficient energy transfer in the mammalian cell is linked to a series of moiety-conserved cycles, including the citric acid cycle. Changes in the cell's environment lead to depletion and replenishment (anaplerosis) of citric acid cycle intermediates. The multiple pathways of

anaplerosis use a variety of substrates, including carbohydrates, odd-chain fatty acids, and amino acids. These pathways reflect a system of redundancy that is important for the functional survival of the cell.

## See Also the Following Articles

ATP Synthesis: Mitochondrial Cyanide-Resistant Terminal Oxidases • Cytochrome *c* • Cytochrome Oxidases, Bacterial • Respiratory Chain and ATP Synthase • Uncoupling Proteins

## Glossary

**anaplerosis** The entry of substrates into the citric acid cycle as intermediates other than acetyl-CoA, thereby increasing the citric acid cycle pool size.

**carboxylation** The introduction of a carboxyl group into a compound.

***β*-oxidation** The oxidative metabolism of fatty acids through a cycle of reactions that removes successive two-carbon units (acetyl-CoA) from the fatty acid.

## Further Reading

Gibala, M. J., Young, M. E., and Taegtmeyer, H. (2000). Anaplerosis of the citric acid cycle: Role in energy metabolism of heart and skeletal muscle. *Acta Physiol. Scand.* **168**, 657–665.

Kornberg, H. (1966). Anaplerotic sequences and their role in metabolism. *Essays Biochem.* **2**, 1–31.

Malloy, C., Sherry, A., and Jeffrey, F. (1988). Evaluation of carbon flux and substrate selection through alternate pathways involving the citric acid cycle of the heart by $^{13}$C-NMR spectroscopy. *J. Biol. Chem.* **263**, 6964–6971.

Martini, W. Z., Stanley, W. C., Huang, H., Rosiers, C. D., Hoppel, C. L., and Brunengraber, H. (2003). Quantitative assessment of anaplerosis from propionate in pig heart *in vivo*. *Am. J. Physiol.* **284**, E351–E356.

Owen, O. E., Kalhan, S. C., and Hanson, R. W. (2002). The key role of anaplerosis and cataplerosis for citric acid cycle function. *J. Biol. Chem.* **277**(34), 30409–30412.

Panchal, A. R., Comte, B., Huang, H., Kerwin, T., Darvish, A., Des Rosiers, C., Brunengraber, H., and Stanley, W. C. (2000). Partitioning of pyruvate between oxidation and anaplerosis in swine hearts. *Am. J. Physiol.* **279**(5), H2390–H2398.

Roe, C. R., Sweetman, L., Roe, D. S., David, F., and Brunengraber, H. (2002). Treatment of cardiomyopathy and rhabdomyolysis in long-chain fat oxidation disorders using an anaplerotic odd-chain triglyceride. *J. Clin. Invest.* **110**, 259–269.

Russell, R., and Taegtmeyer, H. (1991a). Changes in citric acid cycle flux and anaplerosis antedate the functional decline in isolated rat hearts utilizing acetoacetate. *J. Clin. Invest.* **87**, 384–390.

Russell, R., and Taegtmeyer, H. (1991b). Pyruvate carboxylation prevents the decline in contractile function of rat hearts oxidizing acetoacetate. *Am. J. Physiol.* **261**, H1756–H1762.

Taegtmeyer, H., and Passmore, J. M. (1985). Defective energy metabolism of the heart in diabetes. *Lancet* **1**, 139–141.

Vincent, G., Comte, B., Poirier, M., and DesRosiers, C. (2000). Citrate release by perfused rat hearts: A window on mitochondrial cataplerosis. *Am. J. Physiol.* **278**(5), E846–E856.

## Biography

Raymond R. Russell III is an Assistant Professor of Medicine (Cardiology) at Yale University School of Medicine. His laboratory investigates mechanisms of non-insulin-mediated glucose uptake, uncoupling proteins, and metabolic regulation in the heart. He received both his M.D. and his Ph.D. from the University of Texas Health Science Center at Houston in 1991 and served his residency and fellowship at Yale.

Heinrich Taegtmeyer is a Professor at the University of Texas-Houston Medical School, a cardiologist, and a biochemist. His research interests focus on the logic of cardiac metabolism. He received his M.D. from the University of Freiburg in Breisgau, Germany; was a resident on the Harvard Medical Service at the Boston City Hospital; and was a cardiology fellow at the Peter Bent Brigham Hospital. From 1978 to 1981 he studied in the Metabolic Research Laboratory at the University of Oxford (England), under the late Sir Hans Krebs and Regnal Hems, where he earned his D.Phil.

# Angiotensin Receptors

**Tadashi Inagami**
*Vanderbilt University, Nashville, Tennessee, USA*

**Angiotensin (Ang), initially considered as a major pressor substance, is now recognized to mediate numerous physiological and pathophysiological functions. Over the past 70 years, four different active forms of angiotensins, Ang II, Ang III, Ang IV, and Ang (1-7), have been identified. The complexity of their action was multiplied by subtypes of their respective receptors, $AT_{1A}$, $AT_{1B}$, and $AT_2$ for Ang II, $AT_4$ for Ang IV, and Ang (1-7) receptors (Table I). Each subtype of the receptors shows more than one signaling mechanism. This article presents an overview of the formation and structure, and the receptors of Ang's and representative signaling pathways that play very colorful regulatory functions, which cover wide areas such as vasoconstriction, vasorelaxation cardiovascular hypertrophy and remodeling, atherosclerosis, thrombosis, stimulation of mineralocorticoid synthesis and release, facilitation of sympathetic outflow, renal electrolyte metabolism, control of central nervous system in water drinking behavior, blood pressure regulation, memory retention, growth inhibition apoptosis, tissue differentiation, arachidonic acid and prostaglandin formation, and regulation of insulin signals.**

## Angiotensins (Ang) Structure and Formation

### Angiotensin I

All angiotensins are derived from the amino terminus of the ~65 kDa prohormone angiotensinogen via a series of proteolytic cleavage by a variety of proteinases and peptidases. The enzyme that initiates this process is renin. This aspartyl protease is active in pH 6–7, reacts exclusively with angiotensinogen, and cleaves only one singular leucyl peptide bond of the prohormone between residues 10 and 11 generating the decapeptide Ang I (Figure 1). Ang I is hormonally inactive, thus it is prohormone as it has no specific receptor to transmit its signal. The physiological, significance of Ang I appears to confer the high degree of specificity to the angiotensin generating system in plasma or intracellular system in which there are numerous proteins.

### Angiotensin II

The inactive Ang I is activated to the active Ang II by the metallo-endopeptidase, angiotensin I converting enzyme (ACE), which is identical to kininase II. This enzyme was the target for the first most successful drugs for the treatment of renin-dependent hypertension, with a minimum of side effects and complications. Starting with captopril, a series of long acting ACE inhibitors were synthesized and were found to almost completely block Ang II formation. Ang II has two subtypes of receptors, $AT_1$ and $AT_2$. The heptapeptide Ang III is the product of amino terminal cleavage of Ang II by aminopeptidases. Although Ang III is prominent in the neuronal system, it shares receptors, $AT_1$ and $AT_2$, with Ang II. The amino acid sequence of rat $AT_1$ and $AT_2$ are shown in Figure 2.

### Ang (1-7)

Ang (1-7) can be formed mainly by the removal of carboxyl terminal tripeptide from Ang I or of a carboxypeptidase from Ang II. It has been shown to activate phospholipase $A_2$ to release arachidonic acid from phospholipids leading to the formation of prostaglandins.

### Ang IV

Ang IV is the hexapeptide Ang (3-8). It was reported to stimulate the production of plasminogen activator inhibitor to stabilize blood clots and to promote atherosclerosis. Other roles in the brain have been reported.

Whereas receptors for Ang II, Ang III, and Ang (1-7) are G-protein coupled receptors, the receptor isolated and cloned from the adrenal membranes is a single transmembrane type.

## Angiotensin Receptor

### Ang I Binding Protein

Despite the ubiquitous distribution of Ang I binding proteins, no physiological function was found for it.

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Angiotensin Receptors**

| Angiotensins | Receptors | G-proteins coupled |
|---|---|---|
| Ang II | $AT_{1a}$ | $G_{q/11}$, $G_i$, $G_{12/13}$ |
| | $AT_{1b}$ | $G_{q/11}$, $G_i$, $G_{12/13}$ |
| | $AT_2$ | $G_i$ |
| Ang III | The same as Ang II | The same as $AT_1$ and $AT_2$ |
| Ang (1-7) | *Mas* oncogene product | ? |
| Ang IV | $AT_4$/IRAM | Single transmembrane receptor |

## ANG II RECEPTORS

### Receptor Subtypes

Synthesis of a series of variants of Ang II revealed the presence of an Ang II receptor, confirming the earlier finding that there are two types of Ang II receptors, one resistant to dithiothreitol and the other sensitive to the treatment. Inactive variants of Ang II were synthesized to obtain Ang II receptor antagonists. These inhibitor peptides also showed the importance of $Arg^2$, $Tyr^4$, and $Arg^8$ for type 1 Ang II receptors. Particularly, replacement of $Phe^8$ to Ile, Thr, or Ala (saralisine) greatly reduced Ang II receptor signals such as $Ca^{2+}$ response although binding is not significantly affected. However, these peptidic analogs were partial agonists. These peptidic inhibitors did not distinguish two different receptors, $AT_1$ and $AT_2$. We had to wait until the syntheses of nonpeptide inhibitors to obtain indications for the presence of at least two receptor subtypes for Ang II that differ in biochemical and physiological responses, localization, and ontogeny.

Direct proof for the two major receptor subtypes $AT_1$ and $AT_2$ were provided by expression cloning of their cDNAs from rat and bovine tissues. Rodents were found to have three genes: $AT_{1a}$, $AT_{1b}$, and $AT_2$. It is likely that birds, reptiles, and amphibians may also have more than one type of receptor. Rat or mouse murine $AT_{1a}$ and $AT_{1b}$ share a 98% amino acid sequence identity through their respective genes and are located in different chromosomes. They can be distinguished only by Northern blot analysis or *in situ* hybridization of 3′ noncoding sequences, where a sizeable difference occurs in nucleotide sequences. In contrast, $AT_1$ and $AT_2$ share only 34% amino acid sequence homology. Noteworthy is the localization of the $AT_2$ gene in the X chromosome throughout species.

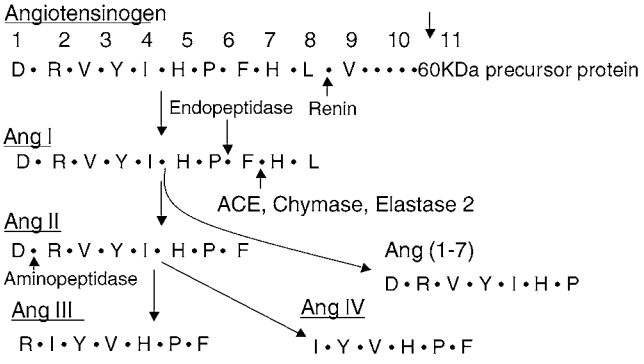



**FIGURE 1** Angiotensinogen and generation.

### $AT_1$ Receptor Signaling

Most of the signal responses considered earlier as Ang II actions seem to be mediated by $AT_1$. These responses are smooth muscle contraction and hypertension, mineralocorticoid synthesis, tyrosine hydroxylase gene expression in adrenal and central and peripheal adrenergic facilitation, stimulation of cellular hypertrophy, mitogenesis and migration, plasminogen activator synthesis, stimulation of fibrosis, renal sodium retentions, centrally regulated dipsogenesis, and hypertension. The major $AT_1$ signaling pathways are mediated by the Gq/11 mediated phospholipase $\beta_1$ activation via cellular [$Ca^{2+}$] increase and protein kinase activation. However, $AT_1$ can also couple to Gi, $G_{12/13}$.

It is now well accepted that $AT_1$ can also activate pathways that involve tyrosine kinase activation such as epidermal growth factor (EGF) receptor through $Ca^{2+}$-stimulated heparin binding-EGF, and consequent activation of Ras, ERK 1/2 which will further result in $p70^{s6k}$ and phosphatidyl inositol-3-kinase and activation of immediate early gene pathways. AT1 also activates the JAK-STAT system, Src family kinases, tyrosine phosphatase SHP-2, PLC-$\delta$, JNK, and p38 MAP kinase. Accumulating lines of evidence seem to support that some of these tyrosine kinase pathways do not involve G-proteins based on results of mutagenesis studies that eliminate key residues required for interaction of cytoplasmic loops of $AT_1$.

Importantly, many of the tyrosine kinase activation steps are activated by the reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide. The formation of super oxide anion is mediated by NAD(P)H oxidase, which is also activated by Ang II. Important recent additions to the knowledge of Ang II mediated smooth muscle contraction is sensitized by the Rho–Rho kinase system. This information is of particular significance for the mechanism of hypertension. On the other hand, cardiovascular hypertrophy and remodeling of resistance vessels are another important aspect for cardiac failure and diminished vascular compliance and hypertension.

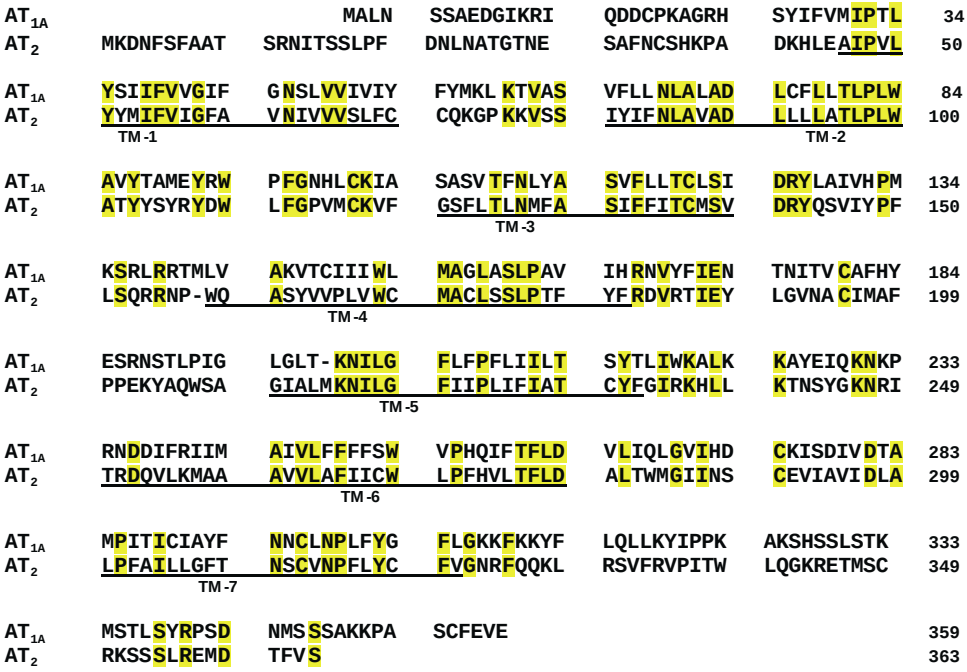



**FIGURE 2** Rat angiotensin receptor $AT_{1A}$ and $AT_2$.

### $AT_2$ Receptor Signaling

Studies on $AT_2$ signaling, particularly using cultured cells, were not straightforward because of rapid disappearance of $AT_2$ receptor during repeated passage of cells and exposure to growth medium. Interpretation of results was also difficult because of rapid ontogenic change in $AT_2$ expression, particularly postnatal decline and increase under stress, during tissue repair or inflammation. *In vivo* studies eliminate many of these difficulties. Thus, *in vivo* studies of loss-of-function studies of $AT_2$ in the kidney, vasculature, heart, and brain particularly adrenal and colon, where $AT_2$ remains expressed in adults, are producing substantive results.

### *In vitro* Studies on $AT_2$ Receptor Signals

Although $AT_2$ (the $AT_2$ receptor) is labile in cells in growth culture medium, some cells, such as PC12w, N1E115, and R3T3, maintain $AT_2$ expression but not $AT_1$ over several generations. Ang II stimulation resulted in growth inhibition via either MAP kinase phosphatase or the tyrosine phosphatase SHP1 even inducing apoptosis, but not all $AT_2$ expressing tissues or cells undergo apoptosis. Ovarian atretic cells express $AT_2$ prominently, yet Ang II treatment failed to induce apoptosis. $AT_2$ expressed in neuronal cells activate Ser/Thr phosphatase PP2A via a Gi-coupled mechanism and was reported to open delayed rectifier $K^+$ channel, a hyperpolarizing mechanism. Its growth inhibitory mechanism on endothelial cells was shown to involve the following sequence of events:

Lowering pH → Release of cycloplasmic kallikrein → generation of bradykinin → activation of NOS

This can explain relaxation or dilation of resistance arteries *ex vivo* by $AT_2$ and its reversal by its specific synthetic inhibitor PD123319 or PD123177 and relaxation by $AT_2$ partial agonists CGP24112. Although $AT_2$ was shown to bind specifically with Gi$\alpha$2 or Gi$\alpha$3, not all of these growth suppressing or cGMP mediated signals were inhibited by pertussis toxin.

### *In vivo* Studies on $AT_2$ Signals

$AT_2$ gene deleted mice and $AT_2$ inhibitor treated mice were shown to slowly retain Na and show impaired natriuresis and markedly diminished urinary cGMP, presumably by the mechanism analogous to endothelial cells discussed above. These observations seem to indicate that the signals mediated presumably by inhibition of phosphatases work in the direction opposite to the growth-stimulating signal of $AT_1$. However, paradoxical observation exists in which $AT_1$ and $AT_2$ function in parallel. An increasing number of publications reported that chronic infusion of the $AT_2$-specific blocker PD123319 was able to inhibit chronic Ang II-induced aortic smooth muscle hypertrophy and fibrosis despite continuously elevated blood pressure. Despite a short half-lifetime of PD123319 *in vivo*, chronic infusion by osmotic mini-pump suppressed

the Ang II-infused aortic hypertrophy. $AT_2$ gene deleted mice also showed marked resistance against left ventricular hypertrophy induced by pressure overload or chronic Ang II infusion. Another example of parallel phenotypic effect of $AT_1$ and $AT_2$ was found in stimulation of tyrosine hydroxylase by both $AT_1$ and $AT_2$ in adrenal chromaffin cells *in vivo*.

Since all of the paradoxical results were obtained under well-controlled conditions, signaling mechanism specific tissues were investigated. It was found that the $AT_2$ binds a zinc finger protein and serves as a transporter to deliver the zinc finger protein to the nucleus, which activated transcription of phosphatidyl inositol 3-kinase p85$\alpha$ subunit (p85$\alpha$PI3K). This triggers a subsequent cardiac hypertrophic pathway. These results indicate that the $AT_2$ mediated cardiac hypertrophy uses signals distinct from that which are activated by $AT_1$. The parallel phenotypic results were due to tissue specific expression of the zinc finger protein in the heart but not in the kidney, where the $AT_2$ signal depends on the kallikrein-NOS-cGMP mechanism. In contrast, the cardiac tissue uses a more direct activation mechanism of nuclear transcription of p85$\alpha$PI3K. The p85PI3K activation has been shown to induce a variety of cardiac hypertrophy mechanisms.

#### Major Physiologic Roles and Morphogenesis of $AT_1$ and $AT_2$

Targeted gene deletion of $AT_{1A}$, $AT_{1B}$, or angiotensinogen in mice showed that angiotensin II has a profound effect on the maintenance of blood pressure, dual deletion of $AT_{1A}$, $AT_{1B}$ resulted in hypotension by 45–50 mmHg and no pressor response to Ang II infusion. $AT_{1B}$ was responsible for only 10% of the pressor effect. Interestingly, these animals showed profound defects in the renal papillary and medullary formation suggesting defects in Na reabsorption, an idea in agreement with the poly urea and increased Na excretion. These animals were stunted and it was difficult to keep them alive without daily saline injection. $AT_2$ gene deletion did not cause overt physical deficiency. However, closer examination revealed that $AT_2$ deficient mice show high frequency (28% penetrance) of uretero-renal pelvis ligation that is closely analogous to a human neonatal disease called Kakkut syndrome. Connection to the ureter of the renal pelvis is the last step of the kidney morphogenesis and $AT_2$ concentration is seen in the renal pelvis at this stage in a normal fetus.

Recently, Srivastava's group reported a very intriguing observation in which they identified families with severe mental retardation and traced their gene defect to the absence or "mis sense" mutation of $AT_2$ gene on the X chromosome. Thus, $AT_2$ function is not only limited to the renal, adrenal, and cardiovascular system, but brain function also depends on $AT_2$. Indeed $AT_2$ gene deficient mice show a pattern of fearful behavior.

## Angiotensin (1-7)

### Functions

Ferrario and his associate reported physiological functions of Ang (1-7). Since the heptapeptide seemed to be generated by one step of carboxypeptidase, it was not clear whether it was a part of Ang II function. However, it was shown that Ang (1-7) is generated from Ang I by an endopeptidase. Since the affinity of the heptapeptide binding to the brain plasma membrane was in the nanomolar range, it seemed to have a physiological significance. Its specific action was shown as arachidonate release and prostaglandin synthesis. Its systemic presence and function were also demonstrated. Decisive evidence of Ang (1-7) as a hormonal peptide came as the receptor was identified.

### Ang (1-7) Receptor

In 1988, Hunley *et al.* presented the hypothesis that *Mas* oncogene product was the Ang II receptor. This hypothesis failed to meet the test of many investigators. $AT_1$ and $AT_2$ were cloned soon afterward as unmistakable Ang II receptors. However, the *Mas* oncogene story made a full circle recently when Santos, Walther, and their collaborators showed that the *Mas* oncogene product expressed in COS7 cells binds Ang (1-7) with a nanomolar $K_D$ value and releases prostaglandins in an abstract form. It has a structural feature of a seven transmembrane domain receptor. Further details are awaited.

## Ang IV

Harding *et al.* showed that the hexapeptide Ang IV binds to receptors in a variety of tissues and increases blood flow. Recently, Xie, Mendelsohn, and their collaborators purified Ang IV receptor from adrenal plasma membrane and succeeded in cloning the cDNA. They found that it is identical with insulin-regulated amino peptidase (IRAP) with a single transmembrane domain. Its possible interaction with insulin is of potential interest as an interface between angiotensin and insulin. However, Ang IV is not the only ligand that binds the extracellular domain. LVV hemorphin, a segment of hemoglobin, was also reported to be a binding ligand. $AT_4$ is expressed heavily in the hippocampus and signals to stimulate acetylcholine. Its potential role in memory retention is of great interest in relation to a possible regulatory role of this aminopeptidase.

Ang II also was shown to be involved in the regulation of long-term potentiation (LTP). Thus, the roles of angiotensin are not limited to the control of blood pressure, water drinking, and dipsogenesis in the hypothalamus and brain stem. $AT_4$ expressed in hippocampus, $AT_2$ expressed in central amygdala nucleus, and Ang II stimulation of LTP points to roles of angiotensins in normal cortical function.

## $AT_3$

We reported non-$AT_1$-non $AT_2$ Ang II receptors in neuro 2A cells were expressed at room temperature, but not at 37 °C. Therefore, we consider it as a likely product of mycoplasm that infected the neuro 2A cell line we used. Thus, at present $AT_3$ is not of mammalian origin.

## See Also the Following Articles

Epidermal Growth Factor Receptor Family • JAK-STAT Signaling Paradigm • Src Family of Protein Tyrosine Kinases

## Glossary

**angiotensin II** Peptide hormone derived from angiotensinogen.
**cardiovascular effects** Effects on the heart and blood vessels.
**receptor** Hormone binding protein.
**receptor signal** Reaction emitted from the receptor upon hormone binding.

## Further Reading

Berk, B. C., and Corson, M. A. (1997). Angiotensin II signal transduction in vascular smooth muscle, role of tyrosine kinase. *Circ. Res.* **80**, 607–616.

deGasparo, M., Catt, K. J., Inagami, T., and Harding, J. (2000). The angiotensin II receptors. *Pharmacol. Rev.* **52**, 639–672.

Eguchi, S., and Inagami, T. (2000). Signal transduction of angiotensin II type 1 receptor through receptor tyrosine kinase. *Regul. Pept.* **91**, 13–20.

Ferrario, C. M., and Iyar, S. M. (1998). Angiotensin (1-7): A bioactive fragment of the renin–angiotensin system. *Regul. Pept.* **78**, 13–18.

Gallinat, S., Busche, S., Raizada, M. K., and Sumners, C. (2000). The angiotensin II type 2 receptor: An enigma with multiple variations. *Am. J. Physiol.* **278**, E357–E374.

Horiuchi, M., Akishita, M., and Dzau, V. J. (1999). Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. *Hypertension* **33**, 613–621.

Inagami, T. (1999). Molecular biology and signaling of angiotensin receptors: An overview. *J. Am. Soc. Nephrology* **10**, 52–57.

Matsubara, H. (1998). Pathophysiological roles of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ. Res.* **83**, 1182–1191.

Timmermans, P. B. M. W. M., Wong, P. C., Chiu, A. T., Herblin, W. F., Carini, D. J., Lee, R. J., Wexler, R. R., Saye, J. A. M., and Smith, R. D. (1993). Angiotensin receptor and angiotensin II antagonists. *Pharmacol. Rev.* **45**, 205–251.

Touyz, R. M., and Schiffrin, E. (2000). Signal transduction mechanisms mediating the physiological and pathological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol. Rev.* **52**, 639–672.

Wright, J. W., Krebs, L. T., Stobb, J. W., and Harding, J. W. (1995). The angiotensin IV system: Functional implication. *Front. Neuroendocrinol.* **16**, 23–52.

## Biography

Tadashi Inagami obtained his B.S. from Kyoto University, Kyoto, Japan in 1953, and his Ph.D. in Chemistry from Yale University in 1958. After his postdoctoral training, he joined the Vanderbilt University School of Medicine and has been on the faculty of the Department of Biochemistry since 1966. He assumed the Stanford Moore Professorship in 1992. His research has centered on the biochemistry of vasoactive substances including renin, angiotensin receptor and signaling on intracellular angiotensin generation, cellular hypertrophy signals in vascular smooth muscle cells, and cardiac myocytes.

# *ara* Operon

**Robert F. Schleif**
*The Johns Hopkins University, Baltimore, Maryland, USA*

**The arabinose operon in the bacterium *Escherichia coli* enables uptake and catabolism of the pentose sugar L-arabinose as the cell's sole source of carbon and energy. In the presence of arabinose, the synthesis of the proteins required for these activities is increased up to 300-fold under the control of the operon's arabinose sensor and regulatory protein, AraC. Expression of the arabinose genes is also subject to catabolite repression, and the catabolite repression protein, CRP, as well as the AraC protein, both bind in the promoter regions of the arabinose gene clusters to regulate expression. In the absence of arabinose, AraC protein binds to two half-sites at the ara $p_{BAD}$ promoter and forms a DNA loop that represses expression. The widespread phenomenon of DNA looping was discovered in the arabinose system. In the presence of arabinose, the loop opens and AraC protein acts positively to assist the binding of RNA polymerase to the $p_{BAD}$ promoter and also speeds the conversion of bound RNA polymerase into the transcriptionally active open complex.**

## Genetics and Physiology

The pathway for the catabolism of L-arabinose in *Escherichia coli* includes two uptake systems and three enzymes for the conversion of arabinose into D-xylulose-5-phosphate (Figure 1). The *araE* gene codes for a low-affinity arabinose symport protein that couples arabinose uptake to proton uptake. The *araF, araG,* and *araH* genes code for three components of a high-affinity transport system that uses ATP as the source of energy. The products of the *araA*, *araB*, and *araD* genes catalyze the isomerization of L-arabinose to L-ribulose, its phosphorylation to L-ribulose-5-phosphate, and its epimerization to D-xylulose-5-phosphate, which then enters the pentose phosphate pathway and whose enzymes are not subject to direct regulation by arabinose. Expression of the *araA, B, D, E, F, G,* and *H* genes as well as a gene of unknown function, *araJ*, is under control of the *araC* gene product. Expression of the *ara* genes is also under control of the catabolite repression protein, CRP. Thus, expression is low in growth medium containing glucose. Arabinose stimulates increased initiation of *ara* operon messenger within 3 s of its addition to a growing culture.

## Transcription Regulation Mechanisms

In the absence of arabinose, the dimeric AraC protein binds to the $araO_2$ and $araI_1$ half-sites, which are 210 bp apart, and forms a DNA loop (Figure 2). The presence of the DNA loop interferes with the binding of RNA polymerase to the $p_{BAD}$ and $p_C$ promoters. Upon the addition of arabinose, AraC protein prefers to bind to the adjacent $I_1$ and $I_2$ half-sites. Occupancy of the $I_2$ half-site stimulates the binding of RNA polymerase to its adjacent binding site and assists RNA polymerase in making the transition to an open complex capable of initiating transcription. Protein–protein contacts important for binding and open complex formation are made between the C-terminal domain of the $\alpha$-subunit of RNA polymerase and activation region three of CRP as well as the two DNA binding domains of AraC. Contacts likely are also made between the DNA-binding domain of the AraC subunit bound to $I_2$ and the $\sigma$-subunit of RNA polymerase.

## Control of DNA Looping

AraC protein shifts from the state of preferring to loop DNA (when arabinose is absent) to the state of preferring to bind to adjacent DNA half-sites (when arabinose is present). The mechanism by which arabinose brings about this change in DNA-binding properties is called the light switch mechanism. In the absence of arabinose, an arm of 18 residues extends from the dimerization-arabinose-binding domain of AraC and binds to the backside of the DNA-binding domain. This arm interaction, plus the covalent connection between the dimerization-arabinose-binding domain and the DNA-binding domain, holds the DNA-binding domains of AraC protein in such an orientation with respect to each other that they are well positioned for DNA looping between $O_2$ and $I_1$. Binding to the adjacent $I_1$ and $I_2$ half-sites would require extensive bending of the protein and breaking of at least one of the two arm-DNA-binding domain interactions.

 © 2004, Elsevier Inc. All Rights Reserved. 

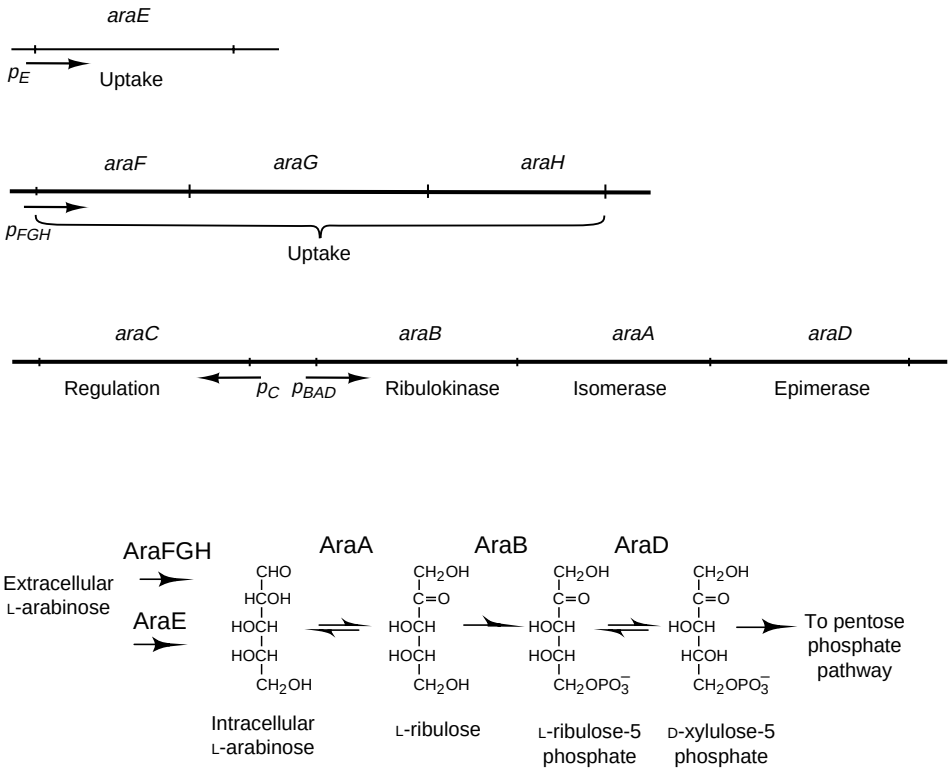



**FIGURE 1** Structure of the *ara* gene clusters in *E. coli* that code for the proteins required for arabinose uptake and catabolism, the catabolic pathway, and the enzymes involved.

When arabinose binds to the dimerization-arabinose-binding domain, the arm prefers to bind over the arabinose rather than to the DNA-binding domain. Thus, the DNA-binding domains are freed from the arm-induced constraints, and they can easily position themselves for binding to the direct repeat half-sites $I_1$ and $I_2$. In this situation, it is energetically more favorable for the protein to bind to these half-sites rather than to form a DNA loop. Thus, the DNA-binding domain that formerly had been bound to $O_2$ repositions to $I_2$, and induction ensues.

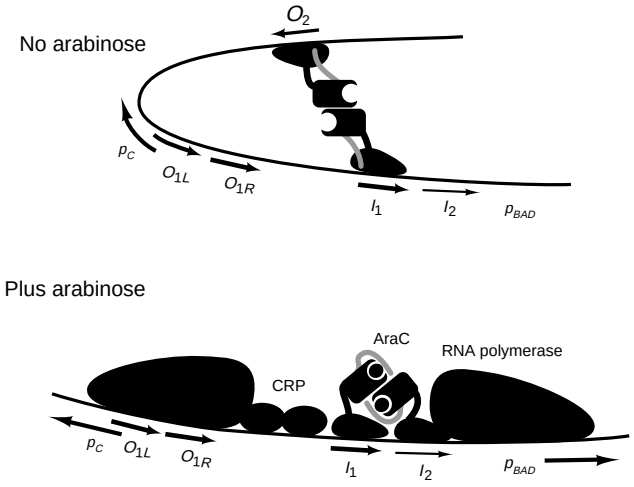



**FIGURE 2** The regulatory region between the *araC* and *araBAD* genes containing the promoters $p_{BAD}$ and $p_C$. In the absence of arabinose, AraC forms a loop between the $O_2$ and $I_1$ half-sites. In the presence of arabinose, AraC binds to the adjacent $I_1$ and $I_2$ half-sites. The N-terminal arm of AraC is indicated in gray. The $p_C$ promoter is considerably less active than the $p_{BAD}$ promoter. Its activity is further depressed by the binding of AraC to the $O_{1L}$ and $O_{1R}$ half-sites.

## The Discovery and Demonstration of DNA Looping

Ellis Englesberg, who initiated genetic studies of the arabinose operon, isolated a deletion extending from outside the *araCBAD* region through at least part of the *araC* gene and into the promoter region. The deletion has the peculiar property that the remaining $p_{BAD}$ promoter can still be fully induced even though the promoter has lost the ability to be repressed by AraC. This property suggested that a site that is required for repression lies upstream from all the sites required for induction. Indeed, an extensive deletion analysis by Schleif confirmed the existence of such repression from upstream and indicated that the upstream site is several hundred nucleotides ahead of all the sites required for induction. The site was definitively identified after the development of genetic engineering technology. *In vivo* footprinting showed

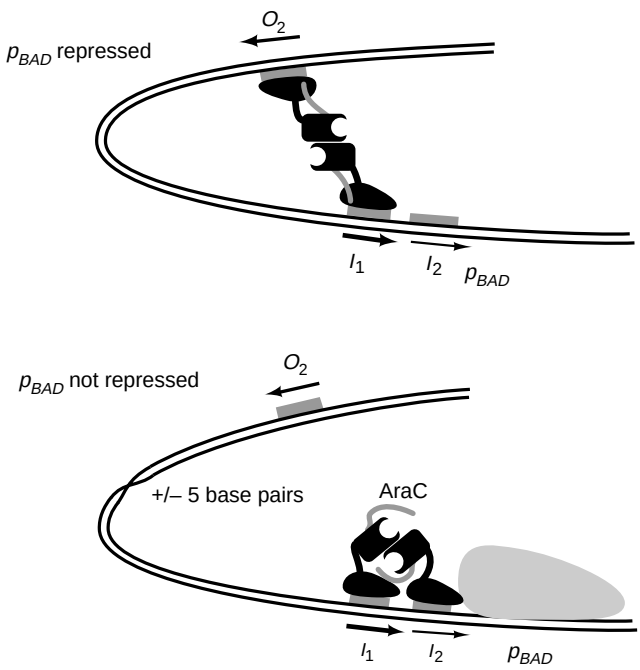



**FIGURE 3** Helical twist experiment showing the involvement of the $O_2$ half-site in repression of $p_{BAD}$. Introduction of 5 bp of DNA between $O_2$ and $I_1$ rotates $O_2$ to the backside of the DNA and prevents loop formation. In this situation, a small fraction of AraC, even though arabinose is absent, binds at $I_1$–$I_2$ and somewhat stimulates the activity of $p_{BAD}$.

that both the upstream half-site, *ara*$O_2$, and the *ara*$I_1$ half-site are occupied by AraC and that mutations in either half-site abolish binding at both half-sites. Introduction of 5 bp of DNA between *ara*$O_2$ and *ara*$I_1$ blocks DNA looping and repression by AraC, but still allows induction of $p_{BAD}$ in the presence of arabinose (Figure 3). A 5 bp insertion rotates the $O_2$ half-site half a turn around the helical DNA with respect to the $I_1$ half-site so that $O_2$ the half-site no longer faces toward $I_1$ and loop formation is not possible. The expression pattern of many additional spacing strains that were generated by inserting and deleting DNA between the half-sites reveals a cyclical pattern of repression with a period of 11.1 bp, thus measuring the helical twist of DNA *in vivo*. DNA looping in the *ara* system has also been demonstrated and studied *in vitro* with gel electrophoresis.

## Related Systems

A number of bacteria – e.g., *Salmonella typhimurium, Citrobacter freundii,* and *Erwinia chrysanthemi* – contain genes with greater than 95% sequence identity to AraC, and thus it can be inferred that they contain arabinose operons that are controlled like that in *E. coli*. *Bacilus subtilis* can utilize arabinose as a source of carbon and energy, but its arabinose operon genes are negatively regulated.

At the amino acid sequence level, no close homologues are yet known to the dimerization domain of AraC. Its structure is known to consist of a jelly roll motif that binds arabinose and a coiled-coil motif that dimerizes the protein. Several homologues – including the rhamnose operon regulators, RhaR and RhaS, and the melibiose operon regulator, MelR – are likely however to possess related structures. Many sequence homologues exist to the DNA-binding domain of AraC. Typically, the level of sequence homology between two members of this family, called the AraC/XylS family, is ~20%. Because these family members are gene regulators and generally act positively, they can be inferred to possess similar structure. The structures of MarA and Rob, two proteins in the family, have been determined. Each consists of two helix–turn–helix motifs joined by a long $\alpha$-helix. This provides an explanation of how each of the two DNA-binding motifs that are found in each DNA-binding domain of the dimeric AraC, contact 17 bp half-sites. For AraC these half-sites are in a direct repeat orientation. The half-sites of RhaR and RhaS, however, are in inverted orientation, illustrating how flexibly the dimerization and DNA-binding domains may be connected in this family.

## History and Historical Significance

Genetic analysis of the arabinose operon was begun by Ellis Englesberg. His studies were among the first to suggest that a regulatory protein could act positively to turn on expression of a gene rather than act negatively to turn off expression as had been shown for the *lac* and lambda phage repressors. His genetic analyses also indicated that AraC protein acted negatively and that at least some of the DNA sequence required for this repression lay upstream of all the sites required for induction of expression. Subsequent genetic analysis by Schleif confirmed this result, and revealed DNA looping as the explanation. Further work by Schleif discovered the light switch mechanism by which arabinose controls the preference of AraC to loop in the absence of arabinose and to bind to adjacent DNA half-sites in the presence of arabinose.

The demonstration of positive regulation by AraC was one of the first indications of the wide diversity of regulatory mechanisms that are utilized in nature. Positive regulation is now known to occur in a great many regulation systems, both prokaryotic and eukaryotic. Similarly, DNA looping is widespread in nature. The discovery of DNA looping came at a convenient time to provide an explanation for action-at-a-distance phenomena, e.g., the way in which enhancers in eukaryotic cells can affect gene expression.

## See Also the Following Articles

RNA Polymerase Reaction in Bacteria • RNA Polymerase Structure, Bacterial • T7 RNA Polymerase

## Glossary

**CRP** or **CAP** A positive regulator of many genes in *E. coli* that is active only in the presence of cyclic AMP.

**DNA looping** The binding of a protein or a complex of proteins to two well-separated DNA sites.

**light switch** Name of the mechanism by which AraC responds to arabinose. In one position of its N-terminal arm of 18 amino acids AraC prefers to form DNA loops, and in the other position of the arm the protein prefers to activate transcription.

**open complex** RNA polymerase bound at a promoter possessing a melted structure of ~14 bases centered at the site at which transcription will begin. RNA polymerase first binds to double stranded DNA in a closed complex, which then opens.

**positive regulation** The regulation mode of a gene that requires the presence and activity of a protein in addition to RNA polymerase for its expression.

## Further Reading

Dunn, T., Hahn, S., Ogden, S., and Schleif, R. (1984). An operator at −280 base pairs that is required for repression of *araBAD* operon promoter: Addition of DNA helical turns between the operator and promoter cyclically hinders repression. *Proc. Natl. Acad. Sci. USA* **81**, 5017–5020.

Englesberg, E., Irr, J., Power, J., and Lee, N. (1965). Positive control of enzyme synthesis by gene C in the L-arabinose system. *J. Bact.* **90**, 946–957.

Greenblatt, J., and Schleif, R. (1971). Arabinose C protein: Regulation of the arabinose operon *in vitro*. *Nat. New Biol.* **233**, 166–170.

Hirsh, J., and Schleif, R. (1973). On the mechanism of action of L-arabinose C gene activator and lactose repressor. *J. Mol. Biol.* **80**, 433–444.

Lobell, R., and Schleif, R. (1990). DNA looping and unlooping by AraC protein. *Science* **250**, 528–532.

Martin, K., Huo, L., and Schleif, R. (1986). The DNA loop model for *ara* repression: AraC protein occupies the proposed loop sites *in vivo* and repression-negative mutations lie in these same sites. *Proc. Natl Acad. Sci. USA* **83**, 3654–3658.

Saviola, B., Seabold, R., and Schleif, R. (1998). Arm-domain interactions in AraC. *J. Mol. Biol.* **278**, 539–548.

Zhang, X., Reeder, T., and Schleif, R. (1996). Transcription activation parameters at *ara* $p_{BAD}$. *J. Mol. Biol.* **258**, 14–24.

## Biography

Robert Schleif is a Professor in the Biology and Biophysics Departments at Johns Hopkins University. His principal research interests are in understanding the fundamental mechanisms that govern the action of proteins, particularly those involved with regulation of gene activity. His laboratory discovered the phenomenon of DNA looping and the light switch mechanism.

# ARF Family

**Gustavo Pacheco-Rodriguez, Joel Moss and Martha Vaughan**
*National Institutes of Health, Bethesda, Maryland, USA*

**ARFs or ADP-ribosylation factors are highly conserved 20-kDa guanine nucleotide-binding proteins found in all eukaryotic cells from *Giardia*, the most primitive existing eukaryote, to primates. No ARFs have been found in prokaryotes, which likewise have a paucity of intracellular organelles. ARFs have critical roles in multiple cellular functions, such as protein secretion, cytoskeletal rearrangements, and signal transduction. Vesicular trafficking in eukaryotes is required for intracellular communication and cargo transportation among organelles. Membrane trafficking involves the formation, translocation, and fusion of vesicles of defined structure and composition, initiated at specific sites on intracellular membranes. Vesicles are constructed and dismantled in a step-wise fashion, beginning with initiation at the donor membrane, formation of the membrane bud, assembly of vesicle cargo, and finally, fission to release the vesicle, followed by its transport, tethering at its destination, uncoating, and fusion with the target membrane. The investigation of ARF actions has contributed significantly to understanding many of those events at a molecular level. Although information regarding the function of ARF6 in actin cytoskeleton and membrane dynamics has expanded rapidly in recent years, the mechanisms of trafficking in endoplasmic reticulum and Golgi compartments are probably still better understood and in more detail.**

## Molecular Characteristics of ARFs

ARFs were first identified, purified, and characterized because of their ability to enhance the cholera toxin-catalyzed ADP-ribosylation of G$\alpha$s, the GTP-binding protein activator of adenylyl cyclase. ARF action in cells requires its controlled alternation between the inactive GDP-bound and active GTP-bound forms. This property allows ARFs to regulate enzyme activities and to participate in the formation and transformation of multi-molecular complexes that effect intracellular transport and cytoskeletal rearrangements.

Mammalian ARFs have been grouped in three classes, with ARF1, 2, and 3 in class I, ARF4 and 5 in class II, and ARF6 in class III. Grouping was based on molecular size, amino acid sequence, and gene structure. The yeast *Saccharomyces cerevisiae* has one class III and two class I ARFs. ARF amino acid sequences are highly conserved among species with $>65\%$ identity among human, yeast, and *Giardia* ARFs. All ARFs contain the stereotypical sequences and specific amino acids that function in GTP binding and hydrolysis. One of the hallmarks of these molecules is the presence of the Rossman fold, a $\beta-\alpha-\beta$ structure responsible for nucleotide binding. The availability of high-resolution structures of ARFs allowed identification of other regions, such as the internal $\beta$-sheet that distinguishes ARFs from other subfamilies of GTPases. The ARF structure also determines the specificity of its interactions with other molecules, and thereby its function. ARF structure differs from those of all other ca. 20-kDa GTPases in having an N-terminal extension that ends with an N-myristoylated glycine. The fatty acid modification, plus the added amphipathic helix at the N-terminus of the ARF protein, facilitates its membrane association through interaction with phospholipids as well as proteins.

## ARF-Related Proteins

In both structure and function, ARFs resemble the several subfamilies of ca. 20-kDa Ras-like GTPases that include Rho, Rac, Rab, and Rap. Each of these proteins, like the ARFs, has special, specific functions in cells. It is notable, however, that many of their actions, effects, and mechanisms of regulation are similar and may even appear to overlap. Perhaps this is because all of these GTPases function by interacting with numerous other molecules, simultaneously or sequentially, in a manner that depends on whether GDP or GTP is bound, to modify their activities or effects.

Among the ARF-related proteins, are ARF-like proteins or ARLs that appear to be more diverse in function and, as a group, differ more in structure than do the ARFs. The 181 amino acid sequences of human ARF1 and ARL1 are, however, 56% identical. There is also ARP (ARF-related protein) and the intriguing 64-kDa ARD1 (ARF domain protein 1), with an 18-kDa ARF domain at the C-terminus.

## ARF Actions

With GTP bound, all of the ARFs are activators of the cholera toxin ADP-ribosyltransferase, which is secreted by *Vibrio cholerae*. They also activate the very similar *Escherichia coli* heat-labile enterotoxin (LT) that causes travelers' diarrhea. Allosteric activation of the cholera toxin A subunit (CTA), which is the ADP-ribosyltransferase enzyme, has been well established *in vitro*. The role of ARF in CTA action on cells however, is less clear. The capacity to activate CTA and LT distinguishes ARFs from other GTPases, including the ARF-like proteins or ARLs. The biochemical and/or catalytic behavior of ARFs can be markedly influenced by their interaction with specific phospholipids.

ARF-GTP can also activate mammalian phospholipase D (PLD) and phosphatidylinositol kinases that metabolize phospholipids critical in cell signaling. PLD-catalyzed hydrolysis of phosphatidylcholine produces phosphatidic acid, a molecule that alters the physical properties of membranes and can also activate specific receptors to initiate signaling. Phosphorylation of phosphatidylinositol 4-phosphate by an ARF-activated kinase likewise contributes to the modification of membrane composition and thus the molecular interactions that can be vitally important in properly controlling cell functions.

The shape of the ARF molecule differs dramatically depending on whether GDP or GTP is bound. Activation of ARF by GTP binding results from movement of the N-terminal helix with its N-myristoylated glycine away from a position close to the surface of the ARF molecule, where it resides when GDP is bound. This facilitates its interaction with other molecules and means that ARF-GTP binds membranes more effectively than does ARF-GDP (Figure 1). Membrane-bound active ARF then recruits adaptor and coat proteins to initiate vesicle formation. ARF function in vesicular trafficking probably involves interactions with phospholipids, as well as with proteins, whether they result in the activation of an enzyme like phospholipase D or in the generation of a vesicle when ARF-GTP becomes membrane-bound to begin the assembly of proteins that will become vesicle coat and cargo. Among the three types of coated vesicles, the COPI- and clathrin-coated structures require ARFs (probably class I ARFs) for formation.

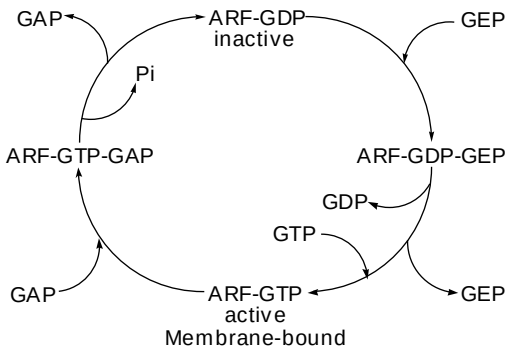



**FIGURE 1** Cycle of ARF activation and inactivation mediated, respectively, by GEPs and GAPs. ARF is active in the GTP-bound form, which is produced by GEP-catalyzed replacement of bound GDP with GTP. Active ARF-GTP associates with membranes, to which it recruits adaptor and coat protein molecules. GAP-accelerated hydrolysis of ARF-bound GTP yields inactive ARF-GDP, which has a lower affinity for membranes and therefore dissociates from them.

## Regulators of ARF Activity

Activation of ARF requires the replacement of bound GDP by GTP, which under physiological conditions occurs very slowly. ARF activation is regulated, therefore, by guanine nucleotide-exchange proteins, or GEPs, which accelerate the replacement process (Figure 1). The turn-off results from hydrolysis of bound GTP, the rate of which is undetectable until ARF-GTP interacts with a GTPase-activating protein or GAP to generate inactive ARF-GDP (Figure 1).

Several families of ARF GEPs with complex regulatory properties that parallel their critical functions have been described. All ARF GEP molecules are characterized by the presence of a ~190-amino acid Sec7 domain, which contains specific sites for ARF binding; also present are residues (i.e., glutamic acid) that act catalytically to accelerate the release of bound GDP and thereby the binding of GTP, which is present at higher concentration in cells than is GDP. Families of GEPs are distinguished by overall molecular size and structural elements outside of the Sec7 domain, as well as by sensitivity to inhibition by brefeldin A (BFA), a fungal fatty acid metabolite. BFA, which was first used experimentally as an inhibitor of viral replication, was later shown to inhibit protein secretion and certain pathways of intracellular membrane trafficking. BFA-inhibited GEPs include mammalian BIG1 and BIG2, yeast Gea1, Gea2, and Sec7, and the *Arabadopsis* GNOM protein.

Among the BFA-insensitive GEPs, the ca. 47-kDa cytohesin family appears to be the most numerous, with four human genes. These molecules contain a central Sec7 domain responsible for ARF activation, followed by a pleckstrin homolog (PH) domain, which binds phosphatidylinositol phosphates with notable specificity; it thereby influences intracellular localization of the protein. The cytohesins are also of interest as cell adhesion molecules. Before its role in ARF activation was recognized, cytohesin-1 had been identified and cloned because of its function in adhesion of lymphocytes to the extracellular matrix. The multiplicity of GEPs (as well as ARFs) complicates delineation of their individual physiological functions, just as the diversity

and complexity of their structures emphasizes the crucial importance to the cell of accurate and timely integration of signals from multiple sources.

There are also several families of GAPs that regulate ARF inactivation, which is just as critical as activation for maintenance of the ARF cyclical function. These proteins contain characteristic zinc-finger structures that are responsible for acceleration of ARF GTPase activity via a critical arginine residue. The diverse GAPs, like the GEP molecules, contain additional structural domains specialized for different functions and interactions, e.g., with cytoskeletal molecules. Three types of ARF GAPs have been recognized: ARF GAP type, GIT type, and ASAP type. Molecules of the last type contain multiple domains, including PH, ankyrin, proline-rich, and SH3. The multiple domains of these proteins enable them to localize at specific intracellular sites and to interact with numerous other molecules with reciprocal effects on function.

## See Also the Following Articles

Phosphatidylinositol Bisphosphate and Trisphosphate • Phospholipase D • Ran GTPase • Rho GTPases and Actin Cytoskeleton Dynamics • Small GTPases

## Glossary

**domain** The element of protein molecular structure, with or without known function, that is conserved among different proteins and/or organisms.

**GAP** GTPase-activating protein that accelerates hydrolysis of GTPase-bound GTP to generate inactive ARF-GDP.

**GEP** Guanine nucleotide-exchange protein that acclerates replacement of GTPase-bound GDP by GTP.

**GTPase** Protein that catalyzes hydrolysis of guanine nucleoside triphosphate (GTP) to yield inorganic phosphate and guanine nucleoside diphosphate (GDP).

**vesicle** Membrane-enclosed intracellular structure, 100 to 200 nm in diameter, with or without visible coat structure, that can be a vehicle for the transport of molecules to support many kinds of cell functions, e.g., secretion, endocytosis, migration, and proliferation.

## Further Reading

Antonny, B., and Schekman, R. (2001). ER export: Public transportation by the COPII coach. *Curr. Opin. Cell Biol.* **13**, 438–443.

Bonifacino, J. S., and Lippincott-Schwartz, J. (2003). Coat proteins: Shaping membrane transport. *Nat. Rev. Mol. Cell. Biol.* **4**, 409–414.

Donaldson, J. G., and Jackson, C. L. (2000). Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.* **12**, 475–482.

Moss, J., and Vaughan, M. (1998). Molecules in the ARF orbit. *J. Biol. Chem.* **273**(34), 21431–21437.

Moss, J., and Vaughan, M. (2002). Cytohesin 1 in 2001. *Arch. Biochem. Biophys.* **397**, 156–161.

Pacheco-Rodriguez, G., Morinaga, N., Noda, M., Moss, J., and Vaughan, M. (2004). Activation of Cholera Toxin and *E. coli* Heat-labile Enterotoxin (LT) by ARF, in press.

Pelham, H. R., and Rothman, J. E. (2000). The debate about transport in the Golgi – Two sides of the same coin? *Cell* **102**, 713–719.

Randazzo, P. A., Nie, Z., Miura, K., and Hsu, V. W. (2000). Molecular aspects of the cellular activities of ADP-ribosylation factors. *Sci. STKE* **21**, 1–15.

Rothman, J. E. (2002). The machinery and principles of vesicle transport in the cell. *Nat. Med.* **8**, 1059–1062.

## Biography

Gustavo Pacheco-Rodriguez, Ph.D., Joel Moss, M.D., Ph.D., and Martha Vaughan, M.D. are members of the Pulmonary-Critical Care Medicine Branch of the National Heart, Lung, and Blood Institute in Bethesda, Maryland. Their research interests include biochemical and molecular mechanisms of ARF function in signal transduction and vesicular trafficking.

# Aspartic Proteases

**Ben M. Dunn**
*University of Florida College of Medicine, Gainesville, Florida, USA*

**Aspartic proteases are one of the four classes of proteolytic enzymes, which cut other proteins into smaller pieces. Proteolytic enzymes are also known as peptidases, because they cleave peptide bonds, and as proteinases, because they cleave proteins. The four classes include serine-, cysteine-, and metallo-proteases, in addition to the aspartic proteases. Each of these classes derives their name from the amino acid residue or functional group in the case of the metallopeptidases, which serves as the critical part of the catalytic mechanism. In the aspartic proteases, it is actually two aspartic acids that make up the catalytic machinery of the enzyme. The carboxylate groups of these two aspartic acid residues assist in proton transfers from a water molecule that acts as the nucleophile, to attack the peptide bond of the peptide or protein to be cleaved. Aspartic proteases are found in all forms of life and play important roles in some disease processes.**

## The Spectrum of Aspartic Proteases in Biology

Historically, aspartic peptidases were first found in the digestive tract of many animals. Pepsin is found in the stomach of most animals with an acidic phase of digestion. A related enzyme, gastricisin, is also present in the stomach and in some animals it is the dominant enzyme. Chymosin, a similar enzyme from the fourth stomach of the calf, is responsible for clotting milk when the young animal suckles from its mother. This helps retain the semisolid form of milk proteins in the digestive track for further processing. The presence of an activity was inferred in ancient times when milk was placed in a sack made from the stomach of an animal so that the milk could be transported on long journeys. The travelers found the milk had clotted and realized that something from the stomach lining was causing this to happen. Chymosin is now used to initiate the production of cheese. In some countries, farmers discovered that certain flowering plants also contained a substance that caused milk to clot and these flowers are used in what is termed artisanal cheese making. Other plant enzymes may be involved in digestion of stored seed protein to provide nourishment for a growing seedling. In the world of fungi, many aspartic peptidases have been found. For example, the common bakers yeast, *Saccharomyces cerevisiae*, contains several enzymes in an intracellular organelle called the food vacuole; one of these enzymes is yeast proteinase A.

Due to the progress of genome sequencing projects from 1990 to the present, sequence information is now available for 354 members of the aspartic protease family. They can be related by sequence identities as seen in Figure 1. Representative examples are given for enzymes from animals, plants, fungi, protozoa, insects, and worms. Furthermore, within one organism, different members of the family play different roles. As an example, humans have two enzymes in the stomach that are involved in the digestion of protein in the diet. In addition, an enzyme in the bloodstream, renin, is involved in regulation of blood pressure by converting a precursor molecule, angiotensinogen to angiotensin I. Subsequent conversion of angiotensin I to angiotensin II has a large effect upon blood pressure, as the latter molecule causes constriction of blood vessels and increases in blood pressure. Within cells, several aspartic proteases are found to play important roles. Cathepsin D in the lysosomes of cells acts to activate other enzymes, prohormones, and growth factors. Cathepsin E, which is found mainly in cells of the lymphoid system, seems to have a function in the response of cells to immunological stimuli. Memapsin, also known as BACE, is involved in the conversion of the $\beta$-amyloid precursor protein to a form that can aggregate and cause fibrils that are found in Alzheimer's disease. Several enzymes known as napsins have recently been discovered in humans. Their function is unknown at this point in time. In the genomes of the plant, *Arabidopsis thaliana*, the nematode, *Caenorhabditis elegans*, and the fruit fly, *Drosophila melanogaster,* a similar pattern of multiple potential functions has emerged, as multiple sequences with identities to the aspartic protease family have also been identified.

## Structure

Pepsin from the stomach of the pig was one of the first proteins to be crystallized; however, the

 © 2004, Elsevier Inc. All Rights Reserved. 

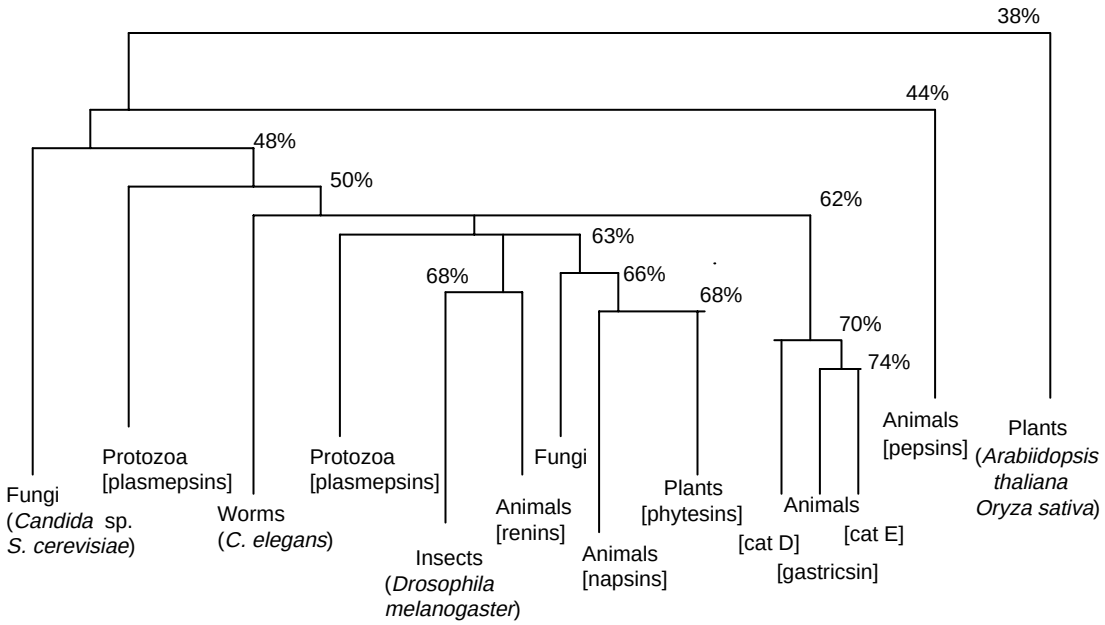



**FIGURE 1** Diagrammatic representation of the relationship among several major classes of aspartic proteinases. The number at the top of each division point is the percentage identity between all species below the line. Organism names are given in italics inside parentheses and specific enzyme names are given inside square brackets. Note that different organisms are seen in different points in the diagram. This is due to the fact that different enzymes within one species play different functional roles.

three-dimensional structure of three fungal enzymes were reported before the structure of pig pepsin was solved. Since the 1970s several hundred structures have been solved by X-ray diffraction. At this point in time, we can state with certainty that all proteins that have sequence homology over 40% with the aspartic protease family will have the same overall structure.

## Overall Structure

The structure of a typical aspartic protease is shown in Figure 2 in several views. The structure can be divided in two ways. First, there is a twofold axis of symmetry down the middle of the protein as seen in Figure 2A. The first ~165 amino acids of the protein (the N-terminal domain) are an independent folding unit that is mostly

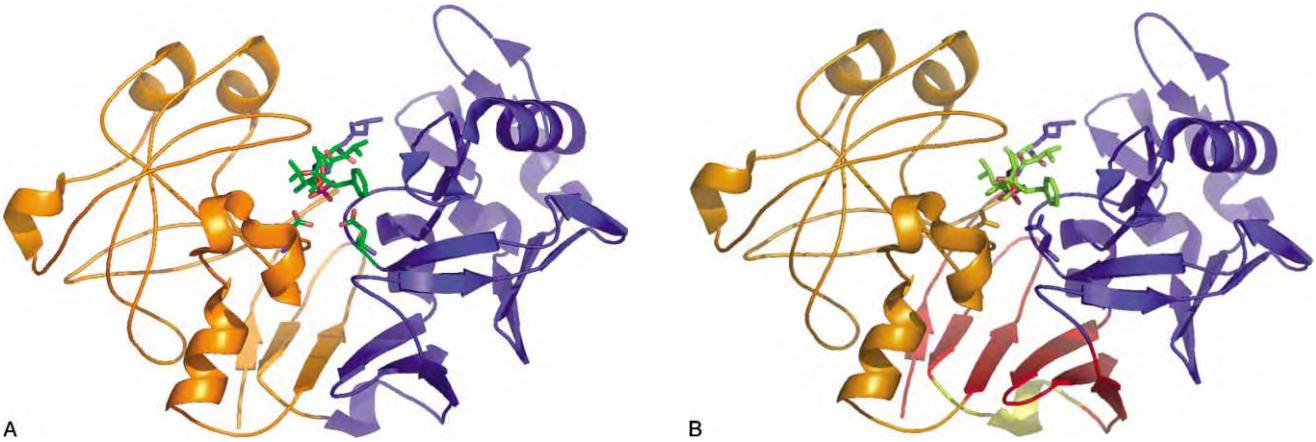



**FIGURE 2** Representation of the three-dimensional structure of a typical aspartic protease. The flat arrows represent $\beta$-strand structure and the spiral shapes represent $\alpha$-helices. These elements are from the backbone only and do not show the complete structure of the protein. (A) View of the enzyme looking end-on into the active site cleft at the top. In this representation, the N-terminal domain is colored orange and the C-terminal domain is colored blue. The two catalytic aspartic acid residues are shown in sticks at the bottom of the active site cleft. Above these amino acids one can see an inhibitor molecule bound in the active site. (B) Same orientation as A, with the N-terminal domain colored orange, the C-terminal domain colored blue, and the $\beta$-sheet at the bottom of the molecule colored red. A small connecting loop between the first three strands of the red $\beta$-sheet and the second three strands is colored yellow.

composed of $\beta$-strands and very little $\alpha$-helical structure. The last ~165 amino acids (the C-terminal domain) are also an independent folding unit with exactly the same folding pattern. The active site cleft is located between the two domains and on one side of the molecule. Each domain contributes to the surface of the cleft, which binds substrates for cleavage or inhibitors that block cleavage. The two halves of the enzymes are believed to have arisen by a gene duplication event before divergence into the different forms shown in Figure 1. A second way to analyze the structure has the same division as described above, but also considers the $\beta$-sheet shown at the bottom of Figure 2B as an independent unit comprised of $\beta$-strands from each of the two domains. In either analysis, all the known structures are highly similar in structure. Some variation is seen in the relative orientation of the two (or three) domains with respect to each other. In addition, some variability is provided by the length of some of the $\beta$-hairpin loops that impinge on the active-site cleft. This provides diversity in the active site binding characteristics of each enzyme, which could be involved in the specific function of each member of the family.

### Catalytic Residues

Each of the two domains (N-terminal and C-terminal) provides one aspartic acid, and these two amino acids are located in the 3D structure at the bottom of the active site cleft, and the carboxylic acid functional group of each of the aspartic acid side chains are within 3–5Å. In the enzyme without bound ligands, a water molecule is hydrogen bonded to the two aspartic acid residues and is the nucleophile that attacks the peptide bond of a substrate held within the active site cleft.

### Flap Structure

Another significant feature of all aspartic proteases is a $\beta$-hairpin that hangs over the active site cleft. This is frequently referred to as the "flap" and movement of this is important in the binding of substrates and inhibitors in the active site. Amino acid differences on the flap, when comparing different members of the family, are important in the different activities exhibited by the enzymes.

### Proenzyme Structure

Most aspartic proteases are synthesized within cells as inactive precursors known as zymogens or proenzymes. In all known cases, this involves an N-terminal extension of between 35 and 125 amino acids. In cases where the 3D structure has been determined for some of the proenzyme forms, the extra amino acid sequence acts to either block the active site cleft or to pull the two domains apart from one another, in either case this prevents activity. For many cases, conversion from the proenzyme to the mature enzyme form is self-catalyzed and occurs when the proenzyme moves from a neutral pH condition to a compartment of lower pH. Removal of the N-terminal extension results in a decrease in molecular weight of between 4 and 15 kDa. In a few known cases, the conversion requires a second enzyme.

## Diseases

Efforts to understand the structure and function of aspartic proteases have been stimulated by the discovery that many disease processes involve the activity of these enzymes.

### Hypertension

As mentioned above, renin is involved in a cascade of reactions that leads to elevation of blood pressure. While this is a normal function that can increase blood pressure in times of activity of an individual, when the levels of renin activity are slightly out of balance, the effects can be dangerous to health. Studies in animals have shown conclusively that giving a selective renin inhibitor, i.e., a compound that will bind to and block the activity of renin without affecting the other aspartic proteases of the human body, has a dramatic effect on blood pressure levels. This area of investigation was extremely important to the study of the whole field of aspartic proteases, as it allowed scientists to develop the tools and strategies to develop selective inhibitors. This has paid dividends in the development of antiviral inhibitors as well as in programs searching for inhibitors against many other family members.

### Cancer

High levels of cathepsin D have been observed in areas surrounding tumors in both humans and animals. This has led to the hypothesis that cathepsin D is involved in the invasiveness of cancer cells. However, cathepsin D activity is normally highest at pH values well below that of tissue. Cathepsin D exhibits its activity in the lysosome, where the pH level is believed to be less than 6. In addition, cathepsin D is released when cells break open, so that the excess levels seen in necrotic tissues may be due to cellular decay. Furthermore, in some conditions, excess levels of cathepsin D are due to overloads of the secretory pathway so that the proenzyme form of cathepsin D is released rather than the mature form. Cathepsin D or the proenzyme form may act as a growth factor to stimulate the growth of cancerous cells.

## AIDS

An aspartic protease is part of the causative agent for AIDS, human immunodeficiency virus. This protease acts to cut apart a viral polyprotein so that new virus particles can assemble. The HIV protease was the second target against which new antiviral drugs were developed. These compounds have had a major impact on therapy for AIDS.

### Malaria

The malaria parasite, *Plasmodium falciparum*, lives during part of its complicated life cycle within human erythrocytes where it degrades hemoglobin for two reasons: one, to provide nutrients in the form of free amino acids for new protein synthesis, and, two, to create space for growth of the parasitic cell. Digestion of hemoglobin is initiated by the action of aspartic proteases. Following the sequencing of the *P. falciparum* genome, it is now known that ten different aspartic protease genes are present. Four of these are expressed and the proteins are located within a special organelle, the food vacuole. This organelle is equivalent to the lysosome of mammalian cells and a similar vacuole of *S. cerevisiae*. Complete digestion of hemoglobin requires the coordinated activity of cysteine proteases and metallo-proteases as well as the aspartic proteases. Inhibitors of aspartic proteases are able to kill the parasite in culture studies.

### Fungal Infections

A number of fungi are known to cause diseases in humans and in plants. For example, *Candida albicans* is a common commensal organism, living within the human body but kept in check by a healthy immune system. In situations where the immune system is compromised, due to chemotherapy for cancer or immune suppression to avoid transplant rejection or due to suppression by diseases such as AIDS, *C. albicans* can become a life-threatening systemic infection. The fungus is also known to have a number of aspartic proteases, including several that are secreted into the extracellular environment surrounding the fungal cells. These enzymes are believed to assist in providing nutrients to the fungus by digesting proteins found in the surrounding environment. Some of these secreted enzymes may work to aid in the invasiveness of the fungus as the infection spreads.

### Alzheimer's Disease

The newest target for drug discovery is the $\beta$-secretase or memapsin. This enzyme acts to cleave the $\beta$-amyloid precursor protein at a specific bond, generating a fragment that can become, with further modification by the so-called gamma secretase, a peptide that forms amyloid fibrils. This condition leads to neurofibrilary plaques in the brain, which are a hallmark of Alzheimer's disease. Due to the unique specificity of memapsin, development of specific inhibitors is possible and new therapies for this condition may develop in the next few years.

## Inhibitors

### Naturally Occurring Inhibitors

Unlike the serine protease family, naturally occurring inhibitors of aspartic proteases are relatively rare. A few have been described in detail and structural information obtained. By investigating the mechanism of inhibition of these protein inhibitors, it is hoped that new insights into inhibitor design will be found.

#### Pepstatin

Culture filtrates of the organism *Streptomyces* have been found to contain many interesting compounds. Pepstatin, discovered around 1970, was found to strongly inhibit pepsin and other members of the aspartic protease family. This compound is a relatively nonspecific inhibitor, as it seems to block most members of the aspartic protease class. Pepstatin contains an unusual amino acid, 3-hydroxy-4-amino-6-methylheptanoic acid. The 3-OH group binds to the two catalytic aspartic acids to form a transition-state analogue, which provides very tight binding. Because all aspartic proteases have the identical catalytic mechanism, pepstatin is an excellent inhibitor. This compound has been used in many studies of the 3D structure of members of the aspartic protease family and, thus provides convenient comparisons between these enzymes. Many synthetic inhibitors, mentioned below, utilize the same concept in the design of selective inhibitors.

#### PI-3 Pepsin Inhibitor of *Ascaris*

The nematode, *Ascaris lumbriocoides*, lives within the human digestive tract for a significant part of the life cycle. The digestive tract contains many proteolytic enzymes, including aspartic proteases. The nematode produces several proteins, each of which is able to bind to and inhibit a specific host enzyme. Several inhibitors have been purified to homogeneity, including PI-3. From a purification of the aspartic protease inhibitors, the third fraction had the highest activity in assays of the ability to inhibit pepsin, thus leading to the designation, PI-3. PI-3 is a protein of 149 amino acid residues. It inhibits pepsins and gastricsins from humans and pigs, and has been shown to inhibit the

cathepsin E from humans. The structure of PI-3 in complex with porcine pepsin has been determined and it was found that the inhibitor is a rigid protein, which makes contact with pepsin at two points, using a total of about 13 out of the 149 residues. The structure of the inhibitor does not change when interacting with pepsin.

### IA-3 Yeast Protease Inhibitor of *S. cerevisiae*

The yeast *S. cerevisiae* synthesizes a 68-residue protein called IA-3, which is a strong and selective inhibitor of yeast proteinase A. The 3D structure of the complex between the two proteins shows that the inhibitor forms a near-perfect $\alpha$-helix between amino acids 2 and 34 when it binds to the enzyme, while the inhibitor when free in solution has little or no organized structure. The IA-3 inhibitor does not inhibit any other member of the aspartic protease class that have been tested so far and, in fact, is cleaved by several of the other enzymes. The transition from an unstructured protein to a tightly folded one is a unique aspect of this inhibitor. This is an example of an "intrinsically unstructured protein."

## Synthetic Inhibitors

Due to the importance of aspartic proteases in several disease processes, strong efforts have gone into the design and testing of compounds that could become new drugs for treatment of disease. In many cases, the observations that pepstatin was a potent inhibitor gave the initial clue to inhibitor design. Thus, the presence of a hydroxyl group within the structure of a new compound is frequently coupled with the variation in the amino acid sequence (for peptide-like compounds), or in the structural groups (for non-peptide compounds) to create a selective inhibitor. Other variations on the central hydroxyl group include replacements for the peptide bond such as –CH2NH–, –PO2–CH2–, and –CHOH–CHOH–. These replacements have proven effective in the development of new inhibitors.

## See Also the Following Articles

Aminopeptidases • Amyloid • Angiotensin Receptors • HIV Protease • Metalloproteases • Secretases

## Glossary

**active site** A portion of a protein where the catalytic amino acids or associated groups are located and where a binding site for substrate exists.

**chemotherapy** Use of compounds to kill disease-causing cells in the human body.

**hemoglobin** The oxygen carrying molecule of the bloodstream found in red blood cells.

**inhibitor** A molecule that binds to the active site of an enzyme to block the catalytic activity.

**proteolytic enzyme** A protein that has the ability to digest other proteins by cleaving peptide bonds to produce smaller fragments.

**substrate** A molecule acted upon by an enzyme to cause a chemical change.

**three-dimensional structure** The detailed arrangement of atoms in space for a molecule, usually determined by X-ray crystallography.

**transition state analogue** A molecule with a chemical structure that resembles the geometry of the state between a substrate and the products of a reaction.

**zymogen** A precursor form of an enzyme, usually larger in size due to the addition of extra amino acid residues; also known as a proenzyme.

## Further Reading

Dunn, B. M. (ed.) (1999). *Proteases of Infectious Agents*. Academic Press, San Diego.

Dunn, B. M. (2002). Structure and mechanism of the pepsin-like family of aspartic peptidases. *Chem. Rev.* **102**, 4431–4458.

James, M. N. G. (ed.) (1998). *Aspartic Proteinases: Retroviral and Cellular Enzymes*. Plenum, New York.

MEROPS—The Peptidase Database. http://merops.sanger.ac.uk.

## Biography

Ben M. Dunn is Distinguished Professor of Biochemistry and Molecular Biology at the University of Florida College of Medicine. He began his career at Florida after earning a B.S. in chemistry at Delaware in 1967 and a Ph.D. at the University of California, Santa Barbara in bioorganic chemistry, two years of postdoctoral work in protein chemistry, and one year as a staff fellow at the NIH. His laboratory studies the enzymatic function of proteases, using kinetics and biophysical techniques including X-ray crystallography.

# ATP Synthesis in Plant Mitochondria: Substrates, Inhibitors, Uncouplers

**Kathleen L. Soole and R. Ian Menz**
*Flinders University, Adelaide, Australia*

**Adenosine triphosphate (ATP) is the energy carrier of the cell and in eukaryotic cells is synthesized via photosynthesis and respiration. Within respiration there is a low level of ATP synthesis associated with glycolysis in the cytoplasm; however, the majority of ATP is synthesized via oxidative phosphorylation which occurs within mitochondria, specifically via the operation of an electron transport chain (ETC) in the inner mitochondrial membrane. In mammals, flux through the respiratory pathway is tightly regulated by the ATP/ADP ratio or adenylate energy charge of the cell. Plants, unlike mammals, must synthesize all of their cellular components. In plants, the presence of a nonphosphorylating pathway in the mitochondrial ETC and an uncoupler protein in the inner membrane overcomes this restriction by adenylate control. In plants, respiration is not only important for energy production but also has a major role in biosynthesis and anabolic reactions.**

## Mitochondrial Electron Transport Chain in Plants

Sucrose and starch are converted to metabolites that feed into the early steps of glycolysis which occurs in the cytoplasm. The end products of glycolysis in plants can be either pyruvate or malate, the latter being formed by the action of PEP carboxylase and malate dehydrogenase, which together bypass pyruvate kinase. Once within the mitochondrial matrix, pyruvate is metabolized by pyruvate dehydrogenase and the enzymes of the citric acid cycle. Being a substrate for mitochondrial malate dehydrogenase, malate can either feed into the citric acid cycle or be metabolized to pyruvate via NAD-malic enzyme. There must be a balance between these processes, since because of the poor forward equilibrium of malate dehydrogenase, the accumulation of oxaloacetate would prevent further malate metabolism. The net result of these reactions is the production of NADH and $FADH_2$ (via succinate dehydrogenase). During a turn of the citric acid cycle, 1 ATP is produced directly by substrate level phosphorylation, not GTP as in mammalian mitochondria. Matrix NADH is oxidized by the ETC and more ATP produced via oxidative phosphorylation.

Oxidative phosphorylation occurs via the interaction of large lipo-protein complexes of the (ETC) and smaller mobile electron carriers found in the inner mitochondrial membrane (Figure 1). NADH is oxidized by complex I, which donates its electrons to a mobile lipophilic electron carrier, ubiquinone. Complex II or succinate dehydrogenase also donates electrons to the ubiquinone pool. Reduced ubiquinone (or ubiquinol) is oxidized by complex III via the Q-cycle and reduces the mobile peripheral protein cytochrome *c*. By interacting with complex IV (cytochrome oxidase), the electrons carried by cytochrome *c* are donated to the terminal electron acceptor, oxygen. During these electron transfer events, protons are translocated from the matrix side of the inner membrane to the inter-membrane space at complexes I, III, and IV, thus establishing the proton motive force (pmf or $\Delta\mu_{H+}$). If ADP is bound to the $F_oF_1$-ATPase in the inner membrane, then protons pass through this complex and the energy within the pmf is used to synthesize ATP. Once ATP is synthesized, it is then exported out of the matrix in exchange for another ADP via the adenine nucleotide translocase. Thus, the flow of electrons and hence oxygen consumption is tightly under control of cellular ADP levels. This is called acceptor or adenylate control.

In plant mitochondria, additional protein complexes are found associated with this ETC. They are distinct from complexes I–IV in that they participate in the transfer of electrons from NADH to oxygen, but do not contribute to the pmf. These enzymes are the alternative or nonphosphorylating NAD(P)H dehydrogenases (NDE and NDI), which donate electrons to the ubiquinone pool and the alternative oxidase (AOX), which accepts electrons from reduced ubiquinone (Figure 1).

 © 2004, Elsevier Inc. All Rights Reserved. 

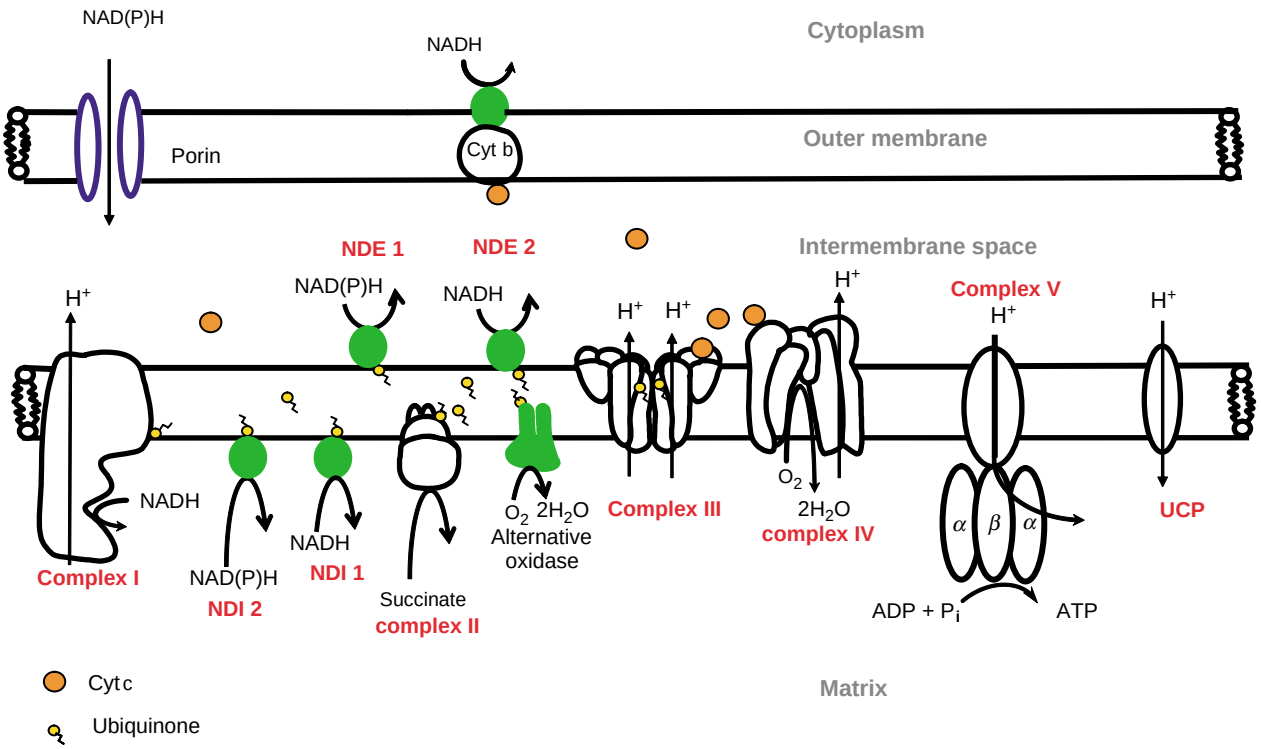



**FIGURE 1** A schematic representation of the routes of NAD(P)H oxidation and the electron transport chain (ETC) of plant mitochondria. NDI refers to internal matrix-facing nonphosphorylating NAD(P)H dehydrogenase, and NDE refers to external cytosolic nonphosphorylating NAD(P)H dehydrogenase. UCP refers to the uncoupling protein. Reproduced from Rasmusson *et al.* (2004) *Annual Review of Plant Biology*, Volume 55.

Evidence for the alternative nonphosphorylating NAD(P)H dehydrogenases comes from the ability of isolated plant mitochondria to oxidize externally supplied NAD(P)H, which cannot pass through the inner membrane. The external cytosolic-facing NAD(P)H dehydrogenase (NDE) feeds directly into the ubiquinone pool, as external NAD(P)H oxidation has an ADP/O ratio equivalent to succinate of less than 2.0. Matrix NADH oxidation in plants has a component which is insensitive to inhibition by the complex I inhibitor, rotenone. This oxidation is catalyzed by NDI. The rotenone-insensitive alternative matrix NADH oxidation has an ADP/O ratio also similar to succinate indicating that NDI also feeds electrons into the ubiquinone pool, bypassing proton pumping at complex I. The alternative oxidase is a cyanide and antimycin A-resistant ubiquinol oxidase that catalyses the reduction of oxygen to water with electrons from ubiquinol and thus bypasses the proton pumps at complexes III and IV.

Thus, it is possible to have electron transport/transfer with no ATP synthesis in plant mitochondria. Therefore, due to the bypasses that exist around key regulatory sites in glycolysis, plant respiration can be totally independent of respiratory control, which gives plants great metabolic flexibility. This flexibility, which results in the loss of adenylate control of respiration by the energy status of the cell can theoretically allow the release of biosynthetic intermediates from the respiratory pathway, independent of energy charge of the cell. This role of the nonphosphorylating pathway is hypothesized and has not been clearly demonstrated *in vivo*.

## Substrates for ATP Synthesis

The plant mitochondrial ETC can oxidize matrix NADH and NADPH to a much lesser extent. The source of this NADH is from pyruvate dehydrogenase and citric acid cycle, and plants have the full complement of these enzymes in addition to other unique enzymes. The presence of matrix pools of NADPH is indicative of the mitochondria's anabolic role. NADP-dependent enzymes involved in folate and thymidylate synthesis such as NADP-dihydrofolate reductase and methylene tetrahydrofolate dehydrogenase have been found in plant mitochondria and their continued operation requires the turnover of the NADPH generated via NDI2. Apart from NAD-dependent isocitrate dehydrogenase, there is also another NADP-dependent form in the matrix, however its role in plant metabolism is not yet clear. Additionally, via the action of a soluble

transhydrogenase detected recently in pea leaf matrix, the mitochondrial oxidation of any strictly NAD-linked substrates such as pyruvate would also be able to produce NADPH.

Another unique enzyme found in plant mitochondria is glycine decarboxylase, which generates NADH in the matrix, is part of photorespiratory cycle and is important in photosynthetic metabolism. It is accepted that the mitochondrial ETC operates in the light, and that ATP synthesis in the light is not exclusively generated from photosynthesis. Evidence indicates that glycine oxidation is linked to a nonphosphorylating NADH dehydrogenase, such as NDI. Recently it has been shown that NDI is regulated at the transcriptional level by light, at least in potato leaf, suggesting a link between nonphosphorylating enzymes and photorespiratory metabolism.

Plant mitochondria also have the capacity to oxidize cytosolic NADH and NADPH via the enzymes NDE1 and NDE2. Thus, ATP synthesis can also occur from the recycling of cytosolic NAD(P)H.

## Inhibitors of ATP Synthesis

There are a multitude of inhibitors that act on specific components of the ETC and the use of many of these has been invaluable in elucidating the composition of the respiratory chain in different species (Table I).

The two most common and specific complex I inhibitors are rotenone and piericidin A, both inhibitors block electron flow between the final iron–sulfur center and ubiquinone. However, they display different inhibition kinetics and it is postulated that the transfer of electrons to quinone may involve more than one quinone-binding site, similar to those observed in complex III. Many other inhibitors of mammalian complex I have been discovered, such as aurachin A and B, thiangazole, phenoxan, and aureothin, and many are likely to inhibit plant complex I; however, their efficacy and specificity has not been demonstrated.

Relatively few inhibitors of plant complex II have been described (Table I), the most widely used is the competitive substrate inhibitor malonate; however, several new complex II inhibitors that are potential fungicides or pesticides have been reported recently.

The two most significant specific complex III inhibitors are antimycin A and myxothiazol, which are specific to the N-side and P-side quinone-binding sites, respectively, and have been important for developing models of the Q-cycle which is involved in proton translocation by complex III.

Complex IV is inhibited by a variety of competitive inhibitors of the oxygen-binding site, such as cyanide and azide. The most interesting of these is the rapid

**TABLE I**

**Specific Inhibitors of Plant Respiratory Enzymes**

| Respiratory protein | Inhibitor | Site/mode of action |
|---|---|---|
| Complex I | Rotenone | Blocks between FeS |
| | Piericidin A | cluster N2 to quinone |
| Complex II | Malonate | Competitive substrate |
| | Thenoyltrifluoroacetone (TTFA) | Blocks FeS cluster S3 |
| Complex III | Antimycin A | Quinone binding (N side) |
| | Myxathiazol | Quinone binding (P side) |
| | Stigamatellin | Quinone binding (P side) |
| Complex IV | Cyanide | Competitive inhibitors of |
| | Azide | $O_2$ binding site |
| | Nitric oxide | |
| Complex V | Oligomycin | Binds to OSCP-subunit |
| | Venturicidin | Proton translocation |
| | | (c-subunit) |
| NDE1 (external NAD(P)H) | DPI | |
| | Calcium chelators | |
| NDE2 (external NADH) | Calcium chelators | |
| NDI1 (internal NADH) | | |
| NDI2 (internal NAD(P)H) | Calcium chelator | |
| | DPI | |
| Alternative oxidase | *n*-Propylgallate | |
| | SHAM | |

and reversible inhibition by nitric oxide, which may play an important role in regulation of oxidative phosphorylation.

There are several chemical inhibitors of ATP synthase (Table I), the most prominent being oligomycin, which is a specific inhibitor of F-type ATP synthases found in a variety of organisms. Apart from the chemical inhibitors, there are also inhibitor proteins, which play a role in regulating the activity *in vivo*. The most characterized is the mammalian inhibitor protein (IF1), whose conformation and inhibitory activity changes in response to pH. Proteins with low homology to IF1 have been found in plants, and although they can inhibit ATP synthetase activity, they do not appear to be regulated by pH. More recently, a class of phosphoserine/phosphothreonine-binding proteins, called the 14-3-3′s, have been identified in plant mitochondria and shown to regulate the ATP synthetase activity.

There are several inhibitors of the alternative NAD(P)H dehydrogenases, while a few show differential selectivity between the various alternative NAD(P)H dehydrogenases many of these such as platanetin, IACA (7-iodo-acridone-4-carboxylic acid), dicumarol, hydroxyflavaones, and sulphydryl reagents can inhibit all of the alternative NAD(P)H dehydrogenases. Interestingly, many of these were originally reported as specific inhibitors of particular alternative NAD(P)H activities. The most effective selective inhibitors are calcium chelators such as EGTA, which are potent inhibitors of the external and NADPH utilizing enzymes, which have a calcium requirement, and DPI (diphenyleneiodonium) which is more effective at inhibiting NADPH utilizing enzymes compared to NADH utilizing enzymes (Table I). SHAM (salicylhydroxamic acid) and *n*-propylgallate are the most prominent inhibitors of the alternative oxidase, the latter being more specific as SHAM can also effect other enzymes of oxidative metabolism such as peroxidases, lipoxygenase, and xanthine oxidases.

## Uncouplers of ATP Synthesis

### Chemical Uncouplers

Oxidative phosphorylation in plants is sensitive to chemical uncouplers such as dinitro phenol (DNP) and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) which dissipate the $\Delta\mu_{H+}$ by carrying proteins across the inner membrane, thus equilibrating the proton gradient. In heterotrophically grown tobacco suspensions cells, cellular respiration is increased by the addition of the uncoupler, FCCP, therefore under these conditions respiration is substrate limited.

### Nonphosphorylating Respiration

Plants have the capacity to uncouple respiration and ATP synthesis using naturally occurring enzymes such as the alternative NDE and NDI, and AOX. Therefore, the level of "coupled" respiration will be dependent on the level and activity of these alternative ETC enzymes.

#### Alternative NAD(P)H Dehydrogenases

In plants, complex I has a much better affinity for NADH than NDI. This suggests that the latter route of electron flow will only be active when the matrix concentration of NADH is high. This has been clearly demonstrated in tissues like potato, which lose NAD from their mitochondrial matrix during longterm storage. In NAD-depleted mitochondria, the rate of electron flow through NDI was markedly reduced, i.e., malate oxidation in isolated mitochondria was totally sensitive to rotenone; however, this could be overcome if the mitochondria were reloaded with NAD from the bathing media. NAD enters via a specific transporter on the inner membrane. It is not clear if this level of regulation occurs *in vivo*. One of the NDI enzymes that uses NADPH as a substrate is calcium-dependent and could be regulated via matrix calcium levels. One question is whether, these alternative pathways operate as overflow for complex I or operate simultaneously during respiration *in vivo*. In a mutant where expression of one of the NDI enzymes was eliminated, the total respiration was reduced, which suggests that NDI contributes to respiration in presence of ADP along with complex I. Both the NDE enzymes are dependent on calcium for maximal activity. It has been suggested that electron flow through the NDE can be regulated by alteration of the local calcium concentration which can be facilitated by polyamines which occur naturally in plant cells, e.g., spermine and spermidine.

#### Alternative Oxidase

For many years, it was thought that the AOX acted as an overflow, only being used when there was an excess of reduced ubiquinone and the cytochrome pathway (via complexes III and IV) was saturated. It is now known that this is not the case, and that AOX and cytochrome pathway can operate simultaneously. Thus, the level of ATP synthesis will rely on the regulation of AOX. AOX can be regulated at both the transcriptional and posttranslational level. AOX exists as a monomer or dimer, with the monomeric form being most active. Further, AOX is activated by organics acids such as pyruvate. Pyruvate is the end product of glycolysis and thus this activation can act as a positive feed-forward mechanism of control when glycolytic flux is high. AOX is induced upon inhibition of the complexes III and/or IV

and also under various environmental stresses. It is thought that the expression of AOX is an attempt by the plant to have a flexible control of ATP synthesis and to maintain growth rate homeostasis. It is also thought to be part of the cell's defense against oxidative stress, as increased flux through the ETC would prevent the accumulation of high-energy electrons in the respiratory pathway, which could react with oxygen to form harmful, reactive oxygen species.

### Uncoupling Protein

In addition to AOX, plants also have uncoupling protein (UCP), which mediates proton re-uptake across the inner membrane (Figure 1). This activity is activated by free fatty acids and inhibited by pyridine nucleotides. UCP is active during respiration in the presence of excess ADP when the free fatty acid concentration is $\sim 4\ \mu M$, and thus can divert energy from oxidative phosphorylation, impacting on the capacity for ATP synthesis. There is a reciprocal regulation between UCP and AOX as free fatty acids inhibit AOX activity. However a precise understanding of the integration and regulation of nonphosphorylating respiratory pathway, UCP and ATP synthase remains a major research challenge.

## See Also the Following Articles

ATP Synthesis: Mitochondrial Cyanide-Resistant Terminal Oxidases • Cytochrome *c* • Cytochrome Oxidases, Bacterial • Respiratory Chain and ATP Synthase • Uncoupling Proteins

## Glossary

**alternative oxidase** A ubiquinol oxidase located in the inner mitochondrial membrane, which accepts electrons from reduced ubiquinone and reduces oxygen to water. During this process, no protons are translocated across the inner mitochondrial membrane.

**nonphosphorylating NAD(P)H dehydrogenase(s)** Enzymes that accept electrons from NADH or NADPH and reduce ubiquinone, a mobile lipophilic electron carrier in the inner mitochondrial membrane with no consequent proton translocation across the membrane.

**nonphosphorylating pathway** A route of electron transfer with no concomitant translocation of protons across the inner mitochondrial membrane, hence this route of electron flow does not contribute to the proton motive force.

**proton motive force** Refers to the proton gradient that is established across the inner mitochondrial membrane during electron transfer through complexes I, II, and IV. Often referred to as $\Delta\mu_{H+}$ or pmf.

## Further Reading

Jarmuszkiewicz, W. (2001). Uncoupling proteins in mitochondria of plants and some microorganisms. *Acta Biochim. Polon.* **48**(1), 145–155.

Joseph-Horne, T., Hollomon, D. W., and Wood, P. M. (2001). Fungal respiration: A fusion of standard and alternative components. *Biochim. Biophys. Acta* **1504**, 179–195.

Moore, A. L., Albury, M. S., Crichton, P., and Affourtit, C. (2002). Function of the alternative oxidase: Is it still a scavenger? *Trends. Plant Sci.* **7**(11), 478–481.

Rasmusson, A. R., Soole, K. L., and Elthon, T. E. (2004). Alternative NAD(P)H dehydrogenases in plant mitochondria. *Annu. Rev. Plant Biol.* **54**, 23–39.

Scheffler, I. E. (2001). Mitochondria make a come back. *Adv. Drug Deliv. Rev.* **49**, 3–36.

## Biography

Dr. Kathleen L. Soole is a Senior Lecturer in the School of Biological Sciences at Flinders University, South Australia. Her principal research interests are in the roles that the nonphosphorylating pathways play in plant mitochondria, plant growth, and stress adaptation. She holds a Ph.D. from the University of Adelaide in South Australia and gained her postdoctoral training at the University of Newcastle-upon-Tyne, England. She has recently identified the gene in Arabidopsis that encodes the protein responsible for matrix nonphosphorylating NADH oxidation in Arabidopsis.

Dr. R. Ian Menz is a Biotechnology Lecturer in the School of Biological Sciences at Flinders University, South Australia. His research interests include the function and structural biology of respiratory proteins. He holds a Ph.D. from the Australian National University, Canberra, Australia. He worked on the structural biology of ATP synthase as a postdoctoral fellow with Sir John E. Walker at the Medical Research Council, Laboratory of Molecular Biology, Cambridge, England.

# ATP Synthesis: Mitochondrial Cyanide-Resistant Terminal Oxidases

**James N. Siedow**
*Duke University, Durham, North Carolina, USA*

**Mitochondria from all higher plants, most algae, many fungi, and some protozoa contain a second terminal oxidase in their electron transfer chain in addition to cytochrome *c* oxidase, the standard terminal oxidase found in all mitochondria. Functionally, this "alternative" oxidase is a ubiquinol oxidase, receiving electrons from reduced ubiquinone and transferring them to oxygen, which is reduced to water. Electron flow from reduced ubiquinone to cytochrome *c* oxidase includes two sites for transporting protons across the inner mitochondrial membrane to form a proton gradient across the membrane that drives ATP formation. Electron flow through the alternative oxidase involves no proton translocation and therefore wastes all the free energy released during electron transfer that would otherwise be conserved in the proton gradient to produce ATP. The role of such an energetically wasteful pathway has yet to be fully elucidated, but in plants it appears to be associated with response to a variety of stresses, possibly acting to prevent over-reduction of the quinone pool and the subsequent formation of harmful reactive oxygen species.**

## The Uniqueness of Plant Mitochondria

Plant mitochondria, like those of all other eukaryotes, contain a standard set of protein complexes that make up the electron transport chain in the inner mitochondrial membrane, complexes I–IV (Figure 1). This electron transfer chain functions as the third stage of aerobic respiration, whereby reductants (NADH, $FADH_2$) generated by operation of the TCA cycle in the mitochondrial matrix are oxidized and the resulting electrons transferred to molecular oxygen. The free energy released during electron transfer is used to translocate protons across the inner membrane from the mitochondrial matrix to the intermembrane space at complexes I, III, and IV, forming an electrochemical proton gradient across the inner membrane. Another inner membrane protein complex (complex V, ATP synthase) provides a path for protons to move back into the matrix and, in the process, couples the energy stored in the proton gradient to the synthesis of ATP from ADP and Pi. The terminal reaction of the electron transfer chain is the reduction of $O_2$ to $H_2O$ by complex IV, cytochrome *c* oxidase. This standard electron transfer pathway is frequently termed the "cytochrome" (cyt) pathway because of the cytochromes present in complexes III and IV and cytochrome *c*. Specific inhibitors of each of the mitochondrial complexes are known. For example, rotenone inhibits complex I, antimycin A inhibits complex III, and cyanide inhibits cyt *c* oxidase.

In addition to the standard cyt pathway, mitochondria in plants contain a variety of additional electron transport proteins that impart flexibility to the system because they can operate without being coupled to ATP synthesis (Figure 1). On the electron input side of the ubiquinone pool are two sets of NAD(P)H dehydrogenases. One pair of dehydrogenases is found on the outside of the inner mitochondrial membrane, allowing mitochondria to oxidize cytoplasmic NADH or NADPH. A second set exists on the inner surface of the inner membrane and oxidizes matrix-derived NAD(P)H. These dehydrogenases do not contribute to proton gradient formation during electron transfer and therefore are able to operate unconstrained by it, unlike proton-pumping components such as complex I whose activity will be impaired as the proton gradient increases. These dehydrogenases are also insensitive to complex I inhibitors such as rotenone. Electrons derived from the oxidation of NAD(P)H by all these dehydrogenases are transferred to the common pool of ubiquinone that also serves as the electron sink for complexes I and II (Figure 1).

## Cyanide-Resistant Respiration

Plant mitochondria also have an additional electron transport branch downstream of the ubiquinone pool due to the presence of a second terminal oxidase in addition to cyt *c* oxidase. This "alternative oxidase"

 © 2004, Elsevier Inc. All Rights Reserved. 

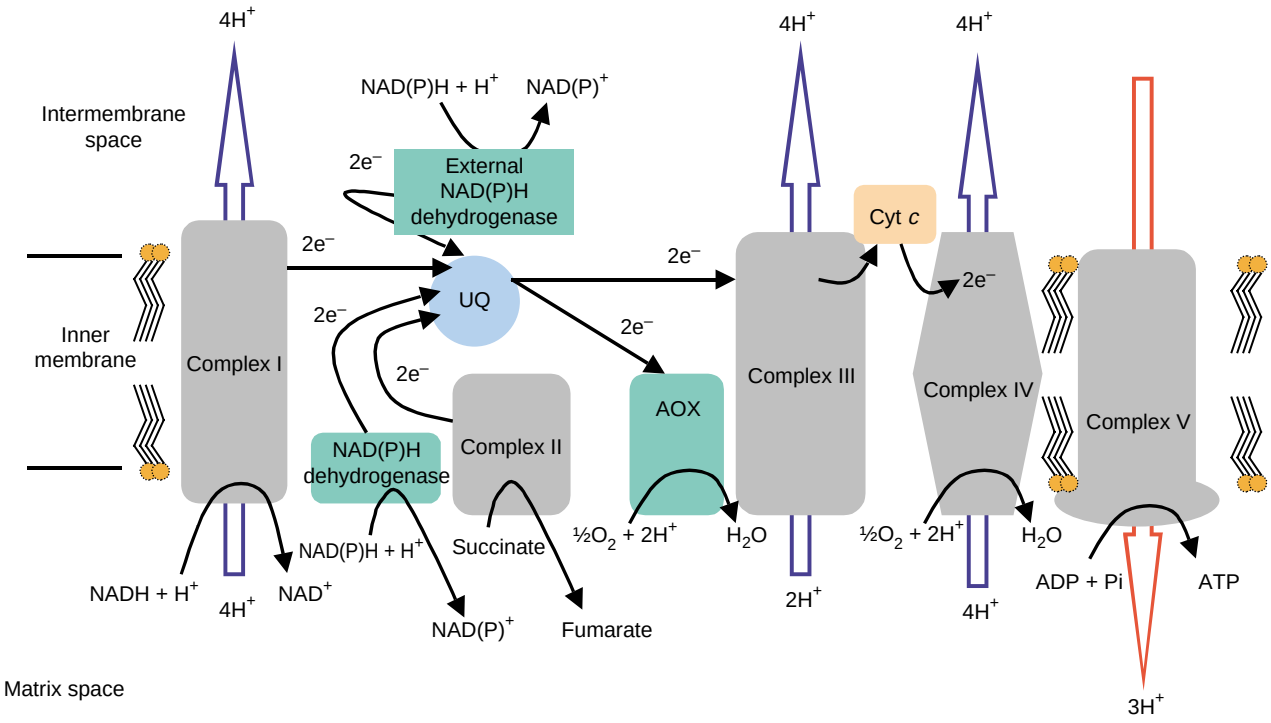



**FIGURE 1** Schematic representation of the plant mitochondrial electron transfer chain.

(AOX) is a ubiquinol:oxygen oxidoreductase that accepts electrons from reduced ubiquinone and subsequently reduces $O_2$ to $H_2O$ (Figure 1). AOX activity is resistant to cyanide and other inhibitors of cyt *c* oxidase but is specifically inhibited by salicylhydroxamic acid (SHAM) and n-alkyl gallate which do not affect the cyt pathway. An important feature distinguishes the two oxidase pathways. Electron flux downstream of the ubiquinone pool through the cyt pathway drives proton gradient formation at complexes III and IV, but no proton gradient formation occurs during operation of the alternative pathway. Thus, AOX would appear to be a wasteful enzyme from an energetic standpoint, eliminating a primary mitochondrial function during aerobic respiration, coupling the oxidation of reductants generated during the operation of glycolysis and the TCA cycle to the synthesis of ATP. Apart from its apparent absence in any metazoan (animal), AOX occurs widely among eukaryotic organisms. AOX has been found in all plants examined to date, but it is also present in most eukaryotic algae, many fungi, and a number of protozoa, including the bloodstream form of the organism responsible for African sleeping sickness, *Trypanosoma bruceii*.

Contemporary understanding of AOX can be traced to the development of an antibody against the plant AOX in 1989. The availability of this antibody led to the first isolation of a plant AOX gene and now dozens of AOX sequences from plants and fungi, and several protozoa and algae are present in gene databases. AOX is nuclear encoded and in plants, it occurs as a small gene family (e.g., five genes in *Arabidopsis*, three in soybean), that produces proteins from 32–35 kDa (~285 amino acids). More recently, characterization of the gene associated with a mutation leading to a defect in chloroplast biogenesis, IMMUTANS, identified a chloroplast protein having sequence similarity to AOX. IMMUTANS also functions as a quinol oxidase, leading to its general characterization as a "plastid terminal oxidase" (PTOX), and it has been shown to participate in a desaturation step during carotenoid biosynthesis in chloroplasts. As prokaryotic genome sequencing has progressed, AOX and PTOX homologues of unknown function have now been identified in an $\alpha$-proteobacterium and several cyanobacteria, respectively. Phylogenetic analyses of the bacterial and eukaryotic sequences suggest that AOX and PTOX arose from a common prokaryotic ancestor, but the two activities diverged prior to the endosymbiotic events that gave rise to mitochondria and chloroplasts.

## AOX Structure

AOX is integral inner membrane protein, but attempts to purify and characterize it have been hampered by the marked instability of the protein following its

solubilization from the membrane and the lack of any apparent visible (>350 nm) absorption spectrum or EPR spectral features in either its oxidized or reduced states. The observation that iron was required for induction of AOX activity in the fungus *Hansenula anomala* originally led to the suggestion that the active site contained iron. AOX sequence analyses later revealed the presence of conserved iron-binding motifs found in members of the family of diiron carboxylate proteins that include the R2 subunit of ribonucleotide reductase and the hydroxylase subunit of methane monooxygenase. This led to the initial development of a model of the AOX active site that contained a four-helical bundle forming a hydroxo-bridged diiron-binding site. The suggestion of a hydroxo-bridged iron center accounted for the absence of any visible absorbance, analogous to the diiron center in methane monooxygenase. Recently, an EPR signal associated with a mixed valence Fe(II)/Fe(III) hydroxo-bridged binuclear iron center has been reported for an *Arabidopsis thaliana* AOX expressed in *Escherichia coli*, providing direct spectral evidence for the diiron carboxylate nature of the AOX (and, by analogy, PTOX) active site.

Over time, the AOX structural model has been refined. As currently envisioned (Figure 2A), the C-terminal two-thirds of the AOX protein comprises a four-helical bundle that makes up the diiron-binding scaffold. Two short hydrophobic helical regions anchor the protein in the inner leaflet of the bilayer, with the bulk of the protein protruding into the mitochondrial matrix. This C-terminal region is relatively conserved among all AOXs, showing 85-90% sequence similarity among plants and over 55% similarity between plants and fungi.

While the C-terminal two-thirds of the AOX protein is responsible for forming the catalytic diiron site, the structure of the N-terminal third of the protein is unknown. When plant and fungal amino acid sequences in this region are compared, there is considerable sequence conservation within each organism type but marked sequence variability between them. Within the N-terminal third of the plant AOX, there is a highly conserved block of ~38 amino acids that includes a regulatory cysteine (*cf.* below). Fungal sequences initially align with plant sequences right after this block. From that point on, the plant and fungal sequences are highly similar with two exceptions: (1) an insertion of 20–25 amino acids unique to fungi just before the first of the four iron-binding helical regions and (2) an extension of the fungal C terminus 20–50 amino acids beyond that found in plants. AOX sequences in protozoa are more similar to the fungal AOX than they are to that of plants.

In plants, chemical cross-linking studies have established that AOX exists in the membrane as a dimer consisting of two AOX monomers (Figure 2B). However, the fundamental unit of catalytic activity based on radiation–inactivation analysis is the monomer. Consistent with the latter observation, AOXs dimers are not universal; cross-linking studies with AOX in both fungi and protozoa have established that they both exist in the membrane as monomers.

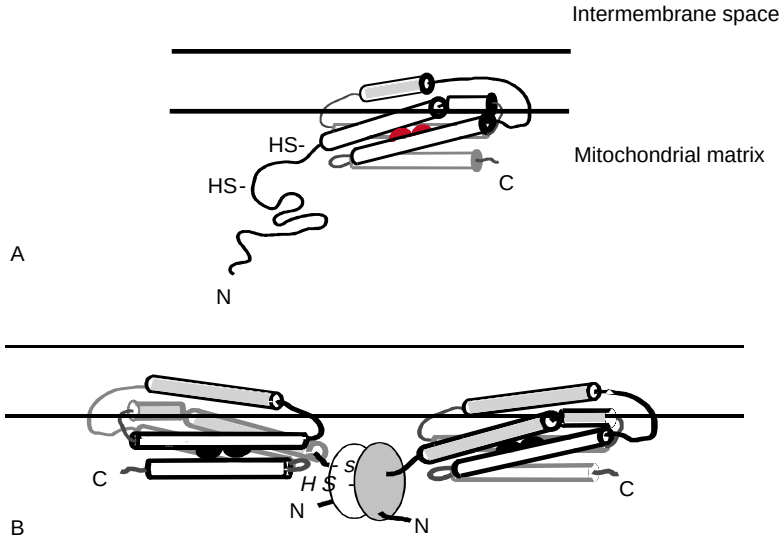



**FIGURE 2** Diagrammatic representation of the structure of the AOX. (A) AOX monomer, (B) dimeric AOX structure found in plants. The two red spheres in (A) represent the diiron center (shown in black in (B)). The inner mitochondrial membrane is delimited by the set of parallel horizontal lines.

## Biochemical Regulation of AOX Activity

While the N-terminal third of the AOX protein is less well characterized structurally than the remainder of the protein, this region has been found to confer regulatory features on the plant AOX. The plant AOX homodimer can exist in two forms, an oxidized state where the two monomeric subunits are covalently linked by an intermolecular disulfide bond and a reduced state where the AOX remains dimeric but the disulfide is reduced to its component sulfhydryls (Figure 3). In the oxidized state, AOX is inactive. When the disulfide is reduced, AOX remains inactive but has the ability to become activated in the presence of an $\alpha$-keto acid, such as pyruvate (Figure 3). In the absence of $\alpha$-keto acids, AOX has essentially no activity. AOX is activated by $\alpha$-keto acids through reaction with a cysteine residue to form a thiohemiacetal. Site-directed mutagenesis of AOX has established that a single cysteine residue (termed CysI, the more N-terminal of two highly conserved cysteines located in the N-terminal region (Figure 2A)) is responsible for both the inactivating disulfide bond and interaction with $\alpha$-keto acids to form the thiohemiacetal that activates AOX (Figure 3). As noted previously, CysI appears in the middle of a conserved block of amino acids in plant AOXs. Given the close proximity of the two regulatory CysIs on adjacent AOX monomers it appears that AOX activation following $\alpha$-keto acid binding results from a conformational change brought about by charge repulsion between the resulting two proximal negative charges.

The regulatory features outlined above provide a facile mechanism for activating AOX under conditions when electron flow through the standard cyt pathway is restricted. Under such conditions, electron transfer would slow down leading to a buildup of reductant in the mitochondrial matrix. The more reduced environment in the matrix would bring about the reduction of the AOX regulatory disulfide, possibly via reduced thioredoxin, which is found in the mitochondrial matrix. As electron transfer becomes limiting, TCA cycle activity would become restricted causing pyruvate concentrations to increase, activating the AOX through a feed-forward regulatory system.

Biochemical regulation of AOX activity in fungi and protozoa is not as well characterized as in plants, but purine nucleotides, particularly GMP, AMP, and ADP, are known to markedly stimulate AOX activity in fungi and protozoa. No stimulation of alternative oxidase activity by $\alpha$-keto acids has been seen in fungi.

## Physiological Role(s) of AOX

The fact that the alternative pathway wastes much of the free energy released during aerobic respiration must be related to its metabolic role. AOX has been found in all higher plants but there is only one instance where its role is clearly established, thermogenesis during flowering in certain members of the plant family Araceae (aroids). Just prior to pollen release, high levels of mitochondria found in club-like structures on the floral inflorescence undergo very high rates of respiration, heating up the inflorescence and volatilizing aromatic compounds that serve to attract pollinating insects. Mitochondria from this thermogenic tissue have extremely high levels of AOX, allowing the high rates of respiration without having to synthesize and use comparable amounts of ATP.

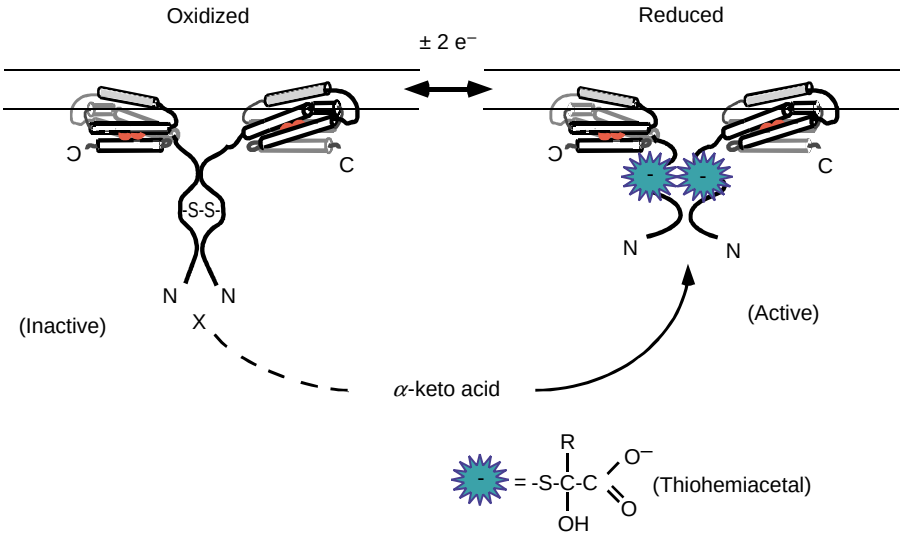



**FIGURE 3** Biochemical regulation of plant AOX by the combined effects of reduction/oxidation of the redox active sulfhydryl/disulfide bond and reaction with $\alpha$-keto acids to form an activating thiohemiacetal.

Most plant tissues do not respire at rates sufficient to support even modest thermogenesis, so that cannot represent its primary function in the vast majority of plant tissues. Because AOX is not involved in proton gradient generation, its activity is independent of the size of the gradient, unlike the cyt pathway, whose activity will be impaired as the proton gradient increases. Therefore, the AOX is in a position to use mitochondrial and cytoplasmic reductant in excess of that needed to maintain ATP synthesis, or when either ATP synthesis or the cyt pathway is impaired. Recognition of this fact originally served as the basis for the suggestion that the alternative pathway only operated when the activity of the cyt pathway was saturated, the so-called "energy overflow" hypothesis. Subsequent studies have established that the alternative pathway can compete with the cyt pathway for electrons from the ubiquinone pool when the cyt pathway is not saturated. This eliminated the overflow hypothesis in is purest form, but not from the standpoint of the alternative pathway helping to regulate mitochondrial electron flow and, as such maintain mitochondrial redox homeostasis.

One outcome of the ability of the alternative pathway to use excess mitochondrial reductant, resulting either from increased input to the ubiquinone pool or decreased use by the cyt pathway, is amelioration of the formation of reactive oxygen species (ROS; superoxide, hydrogen peroxide, hydroxyl radical) by the mitochondrial ubiquinone pool upon over-reduction. Operation of the alternative pathway has been shown to decrease both the reduction state of the ubiquinone pool in roots, and the formation of ROS in plant culture cells. The ability of AOX to decrease mitochondrial ROS formation is the basis of a hypothesis suggesting that AOX plays a role in plant response to environmental stresses. In several circumstances where increased mitochondrial ROS levels are predicted, including chilling stress, post-hypoxia-induced reperfusion injury, and reduced Pi availability, AOX gene expression and protein levels are observed to increase markedly.

Similarly, enhanced AOX gene expression and protein formation have been found to accompany oxidative stresses in fungi, including treatment with hydrogen peroxide. A survey of the appearance of the alternative pathway among yeasts found it was only present in so-called "Crabtree negative" yeast, that lack the presence of alcoholic fermentation as an option to the cyt pathway consistent with the concept of the alternative pathway serving as a mechanism for eliminating excess reducing equivalents in these organisms.

## See Also the Following Articles

ATP Synthesis in Plant Mitochondria: Substrates, Inhibitors, Uncouplers • Cytochrome *c* • Free Radicals, Sources and Targets of: Mitochondria • Mitochondrial Membranes, Structural Organization • Mitochondrial Outer Membrane and the VDAC Channel

## Glossary

**aerobic respiration** The biochemical process whereby reduced organic compounds are completely oxidized in three stages, glycolysis, the TCA cycle, and oxidative phosphorylation with the free energy released used to drive the synthesis of ATP.

**alternative pathway** The mitochondrial electron transfer pathway that goes via a cyanide-resistant, alternative oxidase (AOX) and transfers electrons from the ubiquinone pool to molecular oxygen without storing any of the released free energy in the form of a proton gradient.

**cytochrome (cyt) pathway** The mitochondrial electron transfer pathway that goes from the ubiquinone pool to molecular oxygen via Complex III and Complex IV (cytochrome *c* oxidase) and is coupled at each complex to the transport of protons across the membrane to produce a proton gradient that subsequently drives the synthesis of ATP.

**diiron carboxylate protein** Any member of a large family of proteins that contain a diiron active site formed by a four-helix bundle, two helices of which provide ligands to the two iron atoms via an E–X–X–H sequence motif, and two of which, each provide a single carboxylate ligand (i.e., Glu or Asp) to the irons. Family members include the R2 subunit of ribonucleotide reductase and the hydroxylase subunit of methane monooxygenase.

**reactive oxygen species (ROS)** Any of several highly reactive chemical species that can be formed following the reduction of molecular oxygen ($O_2$), including superoxide anion ($O_2^{\cdot -}$) hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\cdot}$).

## Further Reading

Berthold, D. A., and Stenmark, P. (2003). Membrane-bound diiron carboxylate proteins. *Annu. Rev. Plant Biol.* **54**, 497–517.

Finnegan, P. M., Soole, K. L., and Umbach, A. L. (2004). Alternative mitochondrial electron transport proteins in higher plants. In *Advances in Photosynthesis and Respiration* (D. A. Day, A. H. Millar and J. Whelan, eds.), in press.

Moller, I. M. (2001). Plant mitochondria and oxidative stress: Electron transport, NADPH turnover and metabolism of reactive oxygen species. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 561–591.

Moore, A. L., and Siedow, J. N. (1991). The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. *Biochim. Biophys. Acta* **1059**, 121–140.

Moore, A. L., Albury, M. S., Crichton, P. G., and Affourtit, C. (2002). Function of the alternative oxidase: Is it still a scavenger? *Trends Plant Sci.* **7**, 478–481.

Peltier, G., and Cournac, L. (2002). Chlororespiration. *Annu. Rev. Plant Biol.* **53**, 523–550.

Siedow, J. N., and Umbach, A. L. (2000). The mitochondrial cyanide-resistant oxidase: Structural conservation amid regulatory diversity. *Biochim. Biophys. Acta* **1459**, 423–439.

Vanlerberghe, G. C., and McIntosh, L. (1997). Alternative oxidase: From gene to function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 703–734.

## Biography

James N. Siedow is a Professor in the Department of Biology at Duke University. His research interests are in oxidative processes in plants. He received his Ph.D. from Indiana University and was a Postdoctoral Fellow at Rice University. He has served as an Associate Editor of *Plant Physiology* and *Plant Molecular Biology* and on the Editorial Board of the *Journal of Biological Chemistry* and is a Fellow of the American Association for the Advancement of Science.

# Autophagy in Fungi and Mammals

**Daniel J. Klionsky and Ju Guan**
*University of Michigan, Ann Arbor, Michigan, USA*

**Autophagy refers to the processes by which portions of the cytoplasm are sequestered by membranes and transported into hydrolytic compartments to be degraded. This process occurs by two modes. During macroautophagy, double- or multiple-membrane vesicles, called autophagosomes, form in the cytoplasm. Upon completion, the autophagosome outer membrane fuses with the lysosome/vacuole or endosome. Subsequently the inner membrane and the enclosed cytoplasmic materials are degraded by hydrolases. Microautophagy entails the direct uptake of cytoplasm by invaginations or arm-like extensions of the lysosomal/vacuolar membrane. Autophagy is one of the major mechanisms for degradation and recycling of macromolecules. It is highly regulated by both physiological and environmental cues.**

## Autophagy in Mammals

### Macroautophagy in Mammals

The phenomenon of macroautophagy has been observed in virtually all eukaryotic cell types. Mammalian hepatic tissues and cell cultures have been extensively studied, and in these macroautophagy accounts for the majority of macromolecular recycling once it is induced by hormonal or other signals. The content of the autophagosomes is indistinguishable from its surrounding cytoplasm and often includes recognizable structures such as mitochondria and ribosomes, suggesting that the sequestration is nonselective. There also exists evidence for selective sequestration of certain structures such as peroxisomes, particularly when these organelles are specifically induced to proliferate before the onset of autophagy. Based on morphological and immunocytochemical studies, macroautophagy proceeds through distinct steps of autophagosome formation and maturation (Figure 1). Each of these steps requires ATP.

#### Formation of Autophagosomes

The autophagosomes are distinct from most other intracellular vesicles in that double or multiple delimiting membranes are employed. In addition, formation of these vesicles topologically converts the sequestered cytoplasm to a lumenal or extracellular space. Although the membrane source of the sequestering vesicle has been extensively investigated, the origin is still not known. The autophagosomal membrane may be directly derived from other organelles. Immunostaining of the autophagosomal membrane identified organelle markers of the smooth endoplasmic reticulum (ER), a ribosome-free region of rough ER or the Golgi complex. However, the autophagosomal membranes are extremely poor in proteins, indicating that they do not have protein profiles typical of most endomembranes. Accordingly, it has been proposed that a novel organelle or membrane is dedicated to generating the initial sequestering membrane, termed the phagophore.

#### Maturation of the Phagophore

The maturation of phagophores into autophagosomes proceeds in a stepwise manner. For example, early phagophores may retain protein markers from the membrane of origin. These proteins may be lost and others acquired as the phagophore matures. Upon completion, which is marked by closure of the phagophore membrane, the cytosolic vesicle is termed an autophagosome. The autophagosomes fuse with lysosomes to acquire degradative enzymes. As a result of fusion, the inner autophagosomal membrane is released into the lysosome lumen, where it is broken down to expose the sequestered cytoplasm to the lysosomal hydrolases. The cytoplasmic cargo is subsequently degraded and recycled for use by the cell. A population of autophagosomes may fuse with endosomes to give rise to amphisomes, an intermediate compartment named for its dual role in both macroautophagy and endocytosis. The amphisome ultimately fuses with a lysosome to allow the degradation of its cargo.

#### Regulation of Macroautophagy in Mammals

The regulatory mechanism of macroautophagy is rather complex. Hormones and metabolites may activate or inhibit autophagy in different tissues. The intracellular signaling pathway has been studied mainly by using reagents that can alter the rates of autophagic protein degradation. It is very likely that the cellular responses to

 © 2004, Elsevier Inc. All Rights Reserved. 

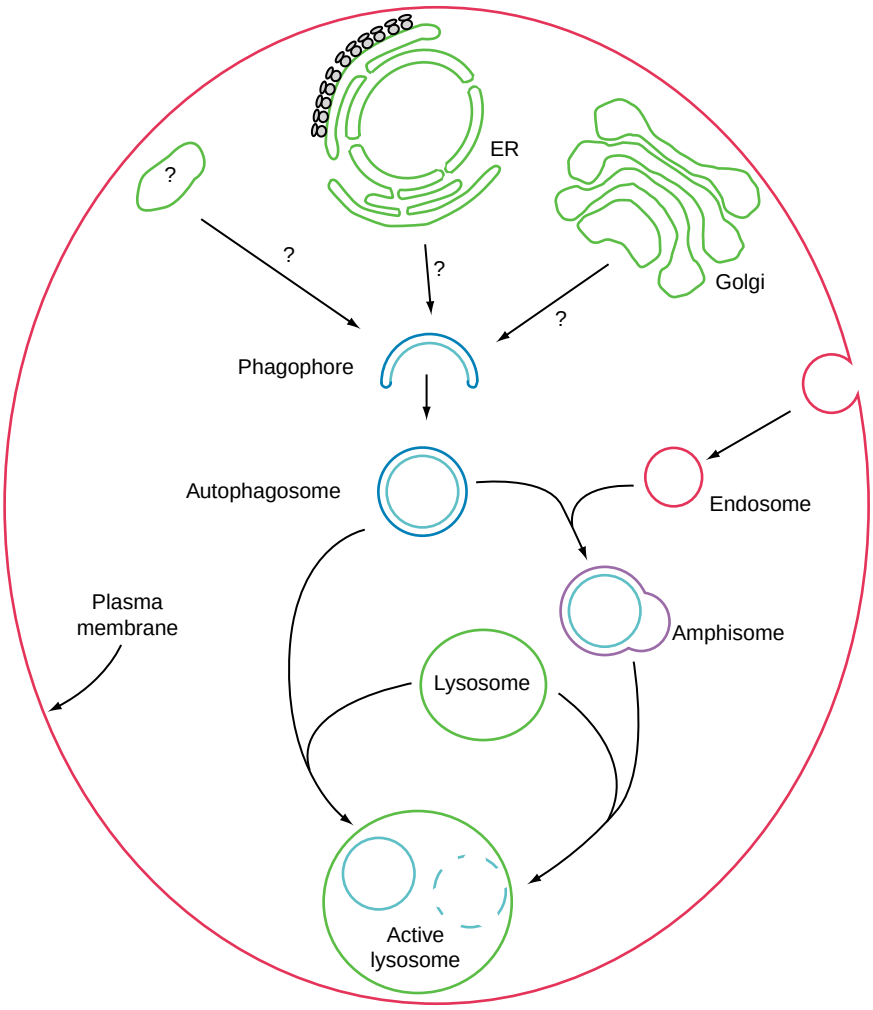



**FIGURE 1** Macroautophagy in mammals. The initial sequestering membrane is termed the phagophore. Upon closure, the resulting double-membrane cytosolic vesicle is called an autophagosome. The origin of the phagophore or autophagosomal membrane is not known, but may include the endoplasmic reticulum, the Golgi complex, or a novel compartment. The autophagosome may fuse with an endosome to form an amphisome. The autophagosome or amphisome ultimately fuses with a lysosome, releasing the inner vesicle that is broken down to allow degradation and recycling of the vesicle contents.

these reagents are pleiotropic. Various proteins have been identified that play a role in regulating macroautophagy, including a phosphatidylinositol 3-kinase, heterotrimeric G proteins, protein kinases, and phosphatases. The inhibition of membrane fusion and cytoskeletal functions affects the final stages of cargo delivery. Table I lists some of the factors and chemicals that affect macroautophagy.

### Microautophagy in Mammals

Microautophagy refers to the direct import of cytoplasmic materials by lysosomal membrane protrusions or invaginations. It is less well characterized in terms of its mechanisms and its contribution to overall protein degradation. In mammals, lysosomes with appendages or intralysosomal vesicles have been observed *in vivo*. Isolated lysosomes have shown the ability to uptake and degrade proteins or incorporate electron-dense markers into intralysosomal vesicles. Microautophagy is also induced by environmental cues such as nutrient deprivation.

## Autophagy in Fungi

### Macroautophagy in Yeast

#### Budding Yeast as a Model for Macroautophagy

During the last decade, autophagy has been studied using the budding yeast *Saccharomyces cerevisiae* as a model system. Nitrogen or carbon starvation induces the formation of cytoplasmic autophagosomes that have the characteristic double delimiting membranes. Similar to

**TABLE I**

**Inhibitors of Macroautophagy in Mammals**

| Compounds | Effects on autophagy | Mechanism/targets |
|---|---|---|
| **Physiological effectors** | | |
| Amino acids | Decrease | Charged tRNA, surface receptors |
| Anabolic hormones (insulin, IGF, EGF) | Decrease | Signaling |
| Catabolic factors/hormones (cyclic AMP, glucocorticoids) | Increase | Signaling |
| **Pharmacological agents** | | |
| Okadaic acid | Decrease | Serine/threonine protein phosphatases |
| Wortmannin, LY294002, 3-methyladenine | Decrease | Phosphatidylinositol 3-kinase |
| Cycloheximide | Decrease | Ribosome |
| Cytochalasin B | Decrease | Microfilaments |
| Colchicine, vinblastine | Decrease | Microtubules |

the findings in mammalian cells, yeast autophagosomal membranes are also protein-poor, as revealed by freeze-fracture electron microscopy studies. Once formed in the cytoplasm, the outer autophagosomal membrane will fuse with the vacuole (equivalent to the mammalian lysosome) membrane, leaving the inner vesicle, called the autophagic body, in the vacuolar lumen. The autophagic bodies are subsequently degraded by vacuolar hydrolases (Figure 2).

Autophagic mutants (*apg*) were isolated that failed to accumulate autophagic bodies when their vacuolar degradation was blocked by a protease inhibitor or by their decreased viability upon nitrogen starvation. An independent group of autophagic mutants (*aut*) were isolated by their inability to degrade a cytoplasmic enzyme under autophagy-inducing conditions.

## The Cytoplasm to Vacuole Targeting Pathway and its Overlap with Macroautophagy

The cytoplasm to vacuole targeting (Cvt) pathway was discovered as the biosynthetic targeting pathway of a vacuolar resident enzyme, aminopeptidase I (Ape1). Precursor Ape1 (prApe1) is synthesized in the cytosol. It rapidly assembles into a dodecamer and remains in an oligomerized form during its course of transport into the vacuole (Figure 2). Cleavage of the N-terminal propeptide of prApe1 by vacuolar proteases gives rise to the mature, active form of the protein (mApe1). A group of *cvt* mutants were isolated that specifically block prApe1 maturation.

Genetic analysis revealed that the *apg* and *aut* mutants overlap with the *cvt* mutants that are defective in prApe1 targeting. Recently, a unified nomenclature has been adopted and the corresponding genes have all been termed autophagy-related (*ATG*). Morphological and biochemical studies further confirmed that prApe1 is transported to the vacuole using a vesicular mode similar to that of macroautophagy. Precursor Ape1 exists in the cytoplasm as a morphological structure that can be detected by electron microscopy. This structure, which consists of multiple prApe1 dodecamers bound to receptor proteins, is called the Cvt complex. Under vegetative growth conditions, the Cvt complex is specifically enwrapped by double-membrane Cvt vesicles. When autophagy is induced, the Cvt complex becomes a preferential cargo of autophagosomes. Thus, uptake of prApe1 represents a type of specific autophagy. Another hydrolase, α-mannosidase, is also targeted to the vacuole via the Cvt and autophagy pathways. The parallel studies of the Cvt pathway complement the analysis of autophagy by providing a specific cargo and the opportunity to study selective uptake mechanisms of autophagy.

## Molecular Mechanism of the Cvt and Autophagic Pathways in Yeast

The molecular machinery was identified by cloning the genes that complement the *atg* mutants. Additional components were found by other approaches, including two-hybrid studies and the identification of homologues to pexophagy genes (see later discussion). Table II lists the Atg proteins and their proposed roles in these pathways. The molecular mechanism for the Cvt pathway and macroautophagy is briefly outlined next.

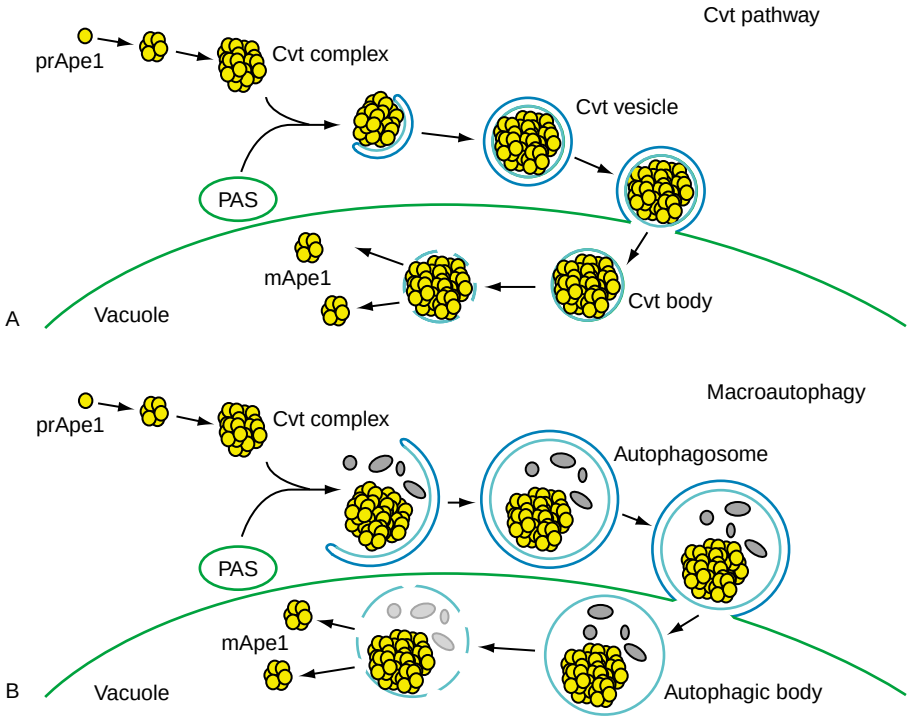



**FIGURE 2** Cytoplasm to vacuole targeting (Cvt) pathway and macroautophagy in yeast. (A) Cvt pathway. Precursor Ape1 (prApe1) forms a dodecamer that assembles into a higher-order Cvt complex. Recruitment of the Cvt complex to the PAS leads to the formation of a Cvt vesicle. The completed Cvt vesicle fuses with the vacuolar membrane and releases the inner vesicle (Cvt body) into the vacuole lumen. Breakdown of the Cvt body allows the maturation of Ape1 (mApe1). (B) Macroautophagy. Under starvation conditions, the PAS is activated by specific signals resulting in the formation of autophagosomes. The Cvt complex is enclosed along with bulk cytoplasm inside autophagosomes. The inclusion of the Cvt complex is a specific process resulting from the action of receptor and adaptor proteins. Fusion between autophagosomes and the vacuole membrane and the subsequent breakdown of the inner membrane (autophagic body) release the cytoplasmic contents into the vacuole lumen for degradation and recycling or, in the case of the Cvt complex, maturation of precursor aminopeptidase I.

**TABLE II**

**Proteins Involved in Autophagy and the Cvt Pathway**

| Function | Proteins | Molecular characteristics |
|---|---|---|
| Organization of the PAS | Atg6, Atg14 | Phosphatidylinositol 3-kinase interacting proteins |
| | Atg9 | Transmembrane protein |
| Localized on PAS; may control initiation of vesicle formation | Atg1, Atg11, Atg13, Atg17, Atg20, Atg24 | Kinase or phosphorylated proteins<br>PX domain proteins bind phosphatidylinositol(3)P |
| Localized on PAS; vesicle formation | Atg5, Atg8, Atg12, Atg16 | Form conjugates or complexes conjugated to phosphatidylethanolamine |
| Conjugating proteins; catalyze formation of Atg12-Atg5 or Atg8-PE | Atg7, Atg3, Atg10, Atg4 | E1 enzyme<br>E2 enzymes<br>Protease |
| Receptor protein of the Cvt complex | Atg19 | Interacts with Atg11 and Atg8 to mediate specific enclosure of cargo |
| Degradation of intravacuolar vesicles | Atg15, Atg22 | Lipase homologue<br>Permease homologue |
| Others | Atg2, Atg18, Atg21, Atg23, Vps51 | Proteins involved in vesicle formation, signaling and/or membrane retrieval |

***A Novel Organelle for De Novo Vesicle Formation*** *In vivo* fluorescence microscopy studies have colocalized the autophagosomal membrane marker protein Atg8 with several other Atg proteins on a perivacuolar structure. This structure is physiologically functional and appears to play a pivotal role in autophagosome formation; therefore, it has been termed the pre-autophagosomal structure (PAS). Localized on the PAS are two conjugates: Atg12, covalently linked to Atg5, and Atg8, covalently attached to phosphatidylethanolamine (Atg8-PE). These conjugates may directly participate in the generation of autophagosomes. Formation of the Atg12-Atg5 and Atg8-PE conjugates involves ubiquitin-like cascades. Recruitment of Atg12-Atg5 and Atg8 to the PAS depends on the function of the transmembrane protein Atg9 and an autophagy-specific phosphatidylinositol 3-kinase complex, underlying the key role of specific lipids in autophagosome formation.

***Regulation of Autophagy*** The Cvt pathway operates under vegetative conditions, whereas autophagy is induced by starvation. Atg1 is localized at the PAS and is important in signaling Cvt vesicle or autophagosome formation, possibly via its differential association with other proteins such as Atg11 and Atg13. In particular, the Atg1-Atg13 interaction is regulated by the TOR (target of rapamycin) kinase. TOR also regulates autophagy at the transcriptional level.

***Mechanism for Cargo Selection in the Cvt Pathway and Autophagy*** The Cvt complex is selectively sequestered by Cvt vesicles or autophagosomes. Atg19, a structural component of the Cvt complex, mediates the recruitment of the oligomerized prApe1 cargo and its recognition by the PAS though protein-protein interactions with Atg11 and Atg8. This may provide a prototype for other selective autophagic targeting pathways.

***Fusion and Breakdown*** The fusion between the Cvt vesicles or autophagosomes and the vacuole membrane is probably similar to vacuole homotypic fusion and requires docking or tethering factors in addition to SNARE and Rab proteins. The breakdown of the inner vesicles requires the action of specific components such as the Atg15 lipase in addition to other vacuolar hydrolases.

## Microautophagy and Selective Organelle Degradation in Yeasts

### Nonselective Microautophagy

The morphological features of microautophagy have been observed in yeasts. Microautophagic vacuole invagination was also reconstituted *in vitro*. Interestingly, the molecular components of macroautophagy, the Atg proteins, were implicated to function at least partly in microautophagy as well.

### Selective Autophagy of Peroxisomes in Methylotrophic Yeasts

Selective degradation of peroxisomes by autophagic mechanisms has been well demonstrated in methylotrophic yeasts. Peroxisomes are necessary for using methanol and are induced when methanol is the sole carbon source. Upon shift of culture medium to preferred carbon sources, superfluous peroxisomes are targeted to the vacuole via either a macro- or microautophagic mode, depending on nutrient conditions. The selective degradation of peroxisomes is termed pexophagy. The proteins involved in glucose-induced selective microautophagy of peroxisomes (Gsa proteins) are generally found to be homologous to the Atg proteins. This finding suggests that macro- and microautophagy may be more closely related at the molecular level than they appear to be morphologically. Alternatively, the Atg proteins may play a unique role in micropexophagy.

## Autophagy, from Yeasts to Mammals

Most of the Atg proteins are conserved in higher eukaryotes, suggesting that the mechanism for autophagy is similar in yeasts and mammals. A few mammalian autophagy proteins have been directly demonstrated to function in macroautophagy. Future studies with mammalian cells may further benefit from the molecular model provided by yeast systems. On the other hand, abnormalities in autophagy have been connected with various human diseases such as Parkinson's disease and certain types of cancer. The importance of autophagy in developmental or pathological macromolecular turnover has yet to be directly demonstrated in higher eukaryotes, but promises to be an exciting area of future research.

## See Also the Following Articles

Aminopeptidases • $G_i$ Family of Heterotrimeric G Proteins • *N*-Linked Glycan Processing Glucosidases and Mannosidases • Peroxisomes • Phosphatidylinositol Bisphosphate and Trisphosphate • Vacuoles

## Glossary

**autophagy** A process in which the cell undergoes membrane rearrangements to sequester a portion of cytoplasm, deliver it to a degradative organelle, and recycle the macromolecular constituents.

**cytoplasm to vacuole targeting (Cvt) pathway** A biosynthetic pathway that transports resident hydrolases to the vacuole through a specific autophagy-like process.

**lysosome** A degradative organelle in mammals that compartmentalizes a range of hydrolytic enzymes and maintains a reduced pH.

**macroautophagy** An autophagic process involving the formation of a double- or multiple-membrane cytosolic vesicle of nonlysosomal or nonvacuolar origin.

**microautophagy** An autophagic process involving direct uptake of cytoplasm at the lysosome or vacuole by protrusion, invagination, or septation of the sequestering organelle membrane.

**pexophagy** A selective type of autophagy involving the sequestration and degradation of peroxisomes.

**vacuole** A degradative organelle in fungi that compartmentalizes a range of hydrolytic enzymes and maintains a reduced pH.

## Further Reading

Blommaart, E. F. C., Luiken, J. J. F. P., and Meijer, A. J. (1997). Autophagic proteolysis: Control and specificity. *Histochem. J.* **29**, 365–385.

Codogno, P., Ogier-Denis, E., and Houri, J.-J. (1997). Signal transduction pathways in macroautophagy. *Cell. Signaling* **9**, 125–130.

Dunn, Jr. W. A. (1994). Autophagy and related mechanisms of lysosome-mediated protein degradation. *Trends Cell Biol.* **4**, 139–143.

Glaumann, H., and Ballard, J. F. (eds.) (1987). *Lysosomes: Their Role in Protein Breakdown*. Academic Press, London.

Kim, J., and Klionsky, D. J. (2000). Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. *Annu. Rev. Biochem.* **69**, 303–342.

Klionsky, D. J. (2004). *Autophagy*. Landes Bioscience, Georgetown, TX.

Klionsky, D. J., and Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science* **290**, 1717–1721.

Klionsky, D. J., and Ohsumi, Y. (1999). Vacuolar import of proteins and organelles from the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **15**, 1–32.

Noda, T., Suzuki, K., and Ohsumi, Y. (2002). Yeast autophagosomes: De novo formation of a membrane structure. *Trends Cell Biol.* **12**, 231–235.

Seglen, P. O., Berg, T. O., Blankson, H., Fengsrud, M., Holen, I., and Stromhaug, P. E. (1996). Structural aspects of autophagy. *Adv. Exp. Med. Biol.* **389**, 103–111.

## Biography

Daniel J. Klionsky is the Abram Sager Collegiate Professor of Life Sciences in the Life Sciences Institute and is a professor in the Departments of Molecular, Cellular, and Developmental Biology and of Biological Chemistry at the University of Michigan, Ann Arbor. His principal research interest is the regulated targeting of proteins and organelles from the cytoplasm into the vacuole using the budding yeast as a model system. He holds a Ph.D. from Stanford University and received his postdoctoral training at the California Institute of Technology. He is a fellow of the John Simon Guggenheim Memorial Foundation and a recipient of the National Science Foundation Director's Award for Distinguished Teaching Scholars.

Ju Guan was a graduate research student in the Klionsky laboratory until 2002. She is currently a postdoctoral research scholar at the University of Michigan, Ann Arbor.

# $B_{12}$-Containing Enzymes

**Vahe Bandarian*** **and Rowena G. Matthews**
*University of Michigan, Ann Arbor, Michigan, USA*

**Vitamin $B_{12}$, or cobalamin, was isolated from liver extracts over 50 years ago as a red crystalline metabolite that cured pernicious anemia in humans. The X-ray crystal structure of the cofactor and various derivatives subsequently showed that it has an elaborate organometallic structure. Cobalamin is widely distributed in nature and biochemical studies have shown that it facilitates enzymatic transformations that involve methyl group transfer and radical-mediated rearrangements. The biosynthesis of cobalamin, the molecular mechanisms of the transformations that are catalyzed by cobalamin-dependent enzymes, and the inherent chemical flexibility in this organometallic cofactor that permit it to participate in these disparate reactions, have fascinated chemists and biochemists for decades. This entry highlights the chemical transformations that are catalyzed by cobalamin-dependent enzymes.**

## The Cobalamin Cofactor

The central feature of the cobalamin cofactor (Figure 1A) is a cobalt atom with octahedral geometry, which is centered in the corrin macrocycle by coordination to pyrrole nitrogens from the corrin in the four equatorial coordination positions of the metal ion. A nitrogen atom that is donated by a dimethylbenzimidazole moiety, which in turn is ligated to the corrin ring, coordinates the cobalt from the lower axial position. In some eubacteria and Archaea, the dimethylbenzimidazole substituent of the cobalamin is replaced by other compounds (e.g., *p*-cresol); such cofactors are referred to as corrinoids. The macrocycle is further adorned by methyl, acetamide, and propionamide side chains. The upper axial position of the cobalt ion is occupied by an alkyl substituent specific to the type of reaction catalyzed by the protein.

The upper face of the cobalamin is where the chemistry takes place in all cobalamin-dependent enzymes. The chemical versatility of cobalamin and corrinoids lies in the carbon–cobalt bond of the cofactor, which is susceptible to cleavage by heterolytic or homolytic pathways (see Figure 1B). The upper coordination position of the cobalamin, in the enzymes that catalyze methyl group transfer, is transiently occupied by a methyl group en route from a donor molecule to an acceptor molecule. In these enzymes, the cobalamin serves both as a nucleophile, accepting the methyl group from the donor, and as a leaving group, donating the methyl group to an acceptor molecule. By contrast, enzymes that catalyze radical-mediated transformations require that the cobalamin have 5′-deoxyadenosine coordinated to the cobalt on the upper face of the corrin. In these enzymes, the organometallic bond between the 5′-methylene of the deoxyadenosyl moiety and the cobalt is severed homolytically to generate a 5′-deoxyadenosyl radical, which initiates the radical cascades that lead to formation of product.

The identity of the ligand that occupies the lower axial coordination position of protein-bound cobalamin may differ significantly from that in solution. In some proteins, in addition to the network of interactions with the side chains of the corrin, the imidazole side chain of a histidine residue substitutes for the dimethylbenzimidazole of the cofactor and donates a nitrogen ligand to the cobalt. This latter form of binding is referred to as a base-off binding mode. Most of the proteins that bind the cobalamin in the base-off mode also contain a DxHxxG sequence that contains the histidine residue that donates the lower axial imidazole ligand. In other proteins, binding of cobalamin to the protein backbone is accomplished by extensive interactions with the corrin and its side chains, and the dimethylbenzimidazole remains coordinated to the cobalt atom. This is referred to as base-on binding of the cofactor.

The cobalamin cofactor can exist in three oxidation states, and each form of the cobalamin exhibits distinct ultraviolet (UV)-visible spectra that allow one to follow the course of the catalytic cycle. For instance, the cobalt in alkyl cobalamins, such as adenosylcobalamin or methylcobalamin, is in the +3 oxidation state (formally, the alkyl ligand is considered to be the anion $R^-$). Homolysis of the cobalamin to generate a deoxyadenosyl radical is accompanied by the formation of a reduced cobalamin, cob(II)alamin, in which the cobalt is in the +2 oxidation state, and reformation of the C–Co bond of the cofactor oxidizes the cobalt to the +3 state. Therefore, adenosylcobalamin-dependent proteins cycle

*Current address: University of Arizona, Tucson, Arizona.

 © 2004, Elsevier Inc. All Rights Reserved. 

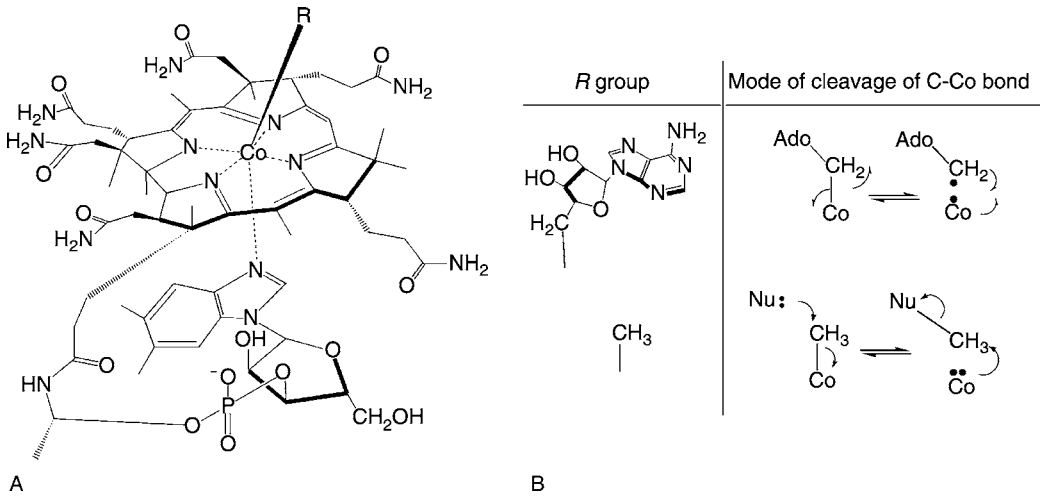



**FIGURE 1** Structure and reactivity of alkylcobalamins. (A) The upper coordination position of cobalamin can be occupied by 5′-deoxyadenosine or by a methyl group. (B) Homolysis of the C–Co bond in adenosylcobalamin-dependent enzymes generates a 5′-deoxyadenosyl radical and cob(II)alamin. By comparison, the cofactor is transiently methylated in enzymes that catalyze group transfer reactions.

between the +3 and +2 oxidation states during the course of the catalytic cycle. By contrast, upon nucleophilic displacement of methyl group from the cobalamin during group transfer chemistry, the cobalamin cycles between the +3 oxidation state in the methylcobalamin form and the +1 state upon loss of the alkyl group. Remethylation of the cobalamin reoxidizes the cobalamin to the +3 oxidation state.

In this entry we summarize the reactions that are catalyzed by cobalamin-requiring enzymes, first highlighting the reactions in which cobalamin serves as a source of the highly reactive 5′-deoxyadenosyl radical and then discussing the reactions that involve methyl transfer chemistry.

## Reactions Catalyzed by Cobalamin-Dependent Enzymes

### Radical-Mediated Rearrangement Reactions

The enzymes that catalyze radical-mediated transformation exploit the inherent weakness of the carbon-cobalt bond of adenosylcobalamin (bond dissociation energy $\sim 30$ kcal $mol^{-1}$) to form the highly reactive 5′-deoxyadenosyl radical. Binding of the cobalamin to these enzymes accelerates the rate of homolysis of the C–Co bond $\geq 10^{12}$-fold. Homolysis of the carbon–cobalt bond is triggered by the presence of the substrate or by an allosteric effector. Presumably, the binding energy derived from substrate/effector-enzyme interactions is essential to elicit the conformational changes that are required to facilitate homolysis of the relatively weak organometallic bond. The 5′-deoxyadenosyl radical is a primary radical and readily abstracts a hydrogen atom from the substrate or from a residue on the protein. Therefore, a hallmark of catalysis by the enzymes in this group is formation of radical intermediates. Figure 2 shows the general catalytic cycles for these enzymes.

Adenosylcobalamin-dependent enzymes can be divided into four groups based on the details of the catalytic transformations (Table I). The reactions catalyzed by enzymes in each group are discussed next.

#### Migration and Elimination Reactions

The enzymes in this group participate in the fermentation of short-chain organic compounds such as ethanolamine, glycerol, 1,2-propanediol, and 1,2-ethanediol. The reactions catalyzed by these enzymes are formally the interchange of a hydrogen atom on one carbon with a group X ( = OH or $NH_3$) at the adjacent position. The catalytic mechanism of these enzymes is quite similar to the mechanism of the carbon skeleton mutase enzymes discussed later. These two groups, in fact, differ only in that the mutases retain the rearranged substituent.

The catalytic mechanisms of ethanolamine ammonia-lyase and diol dehydratase have been studied extensively and a generalized mechanism for these enzymes is shown in Figure 2A. As with all cobalamin-dependent enzymes, the carbon–cobalt bond of the cofactor is homolyzed to generate 5′-deoxyadenosyl radical and cob(II)alamin in the first step of the catalytic reaction. The deoxyadenosyl radical abstracts a hydrogen atom from the substrate (S–H) to generate a substrate-like radical (S·) $\sim$ 9–12Å away from the cob(II)alamin. The rearrangement of S· to a product-like radical (P·) followed by return of a hydrogen atom from the 5′-deoxyadenosine results in the formation of the enol form of the product (P–H). The deoxyadenosyl radical recombines with cob(II)alamin to regenerate

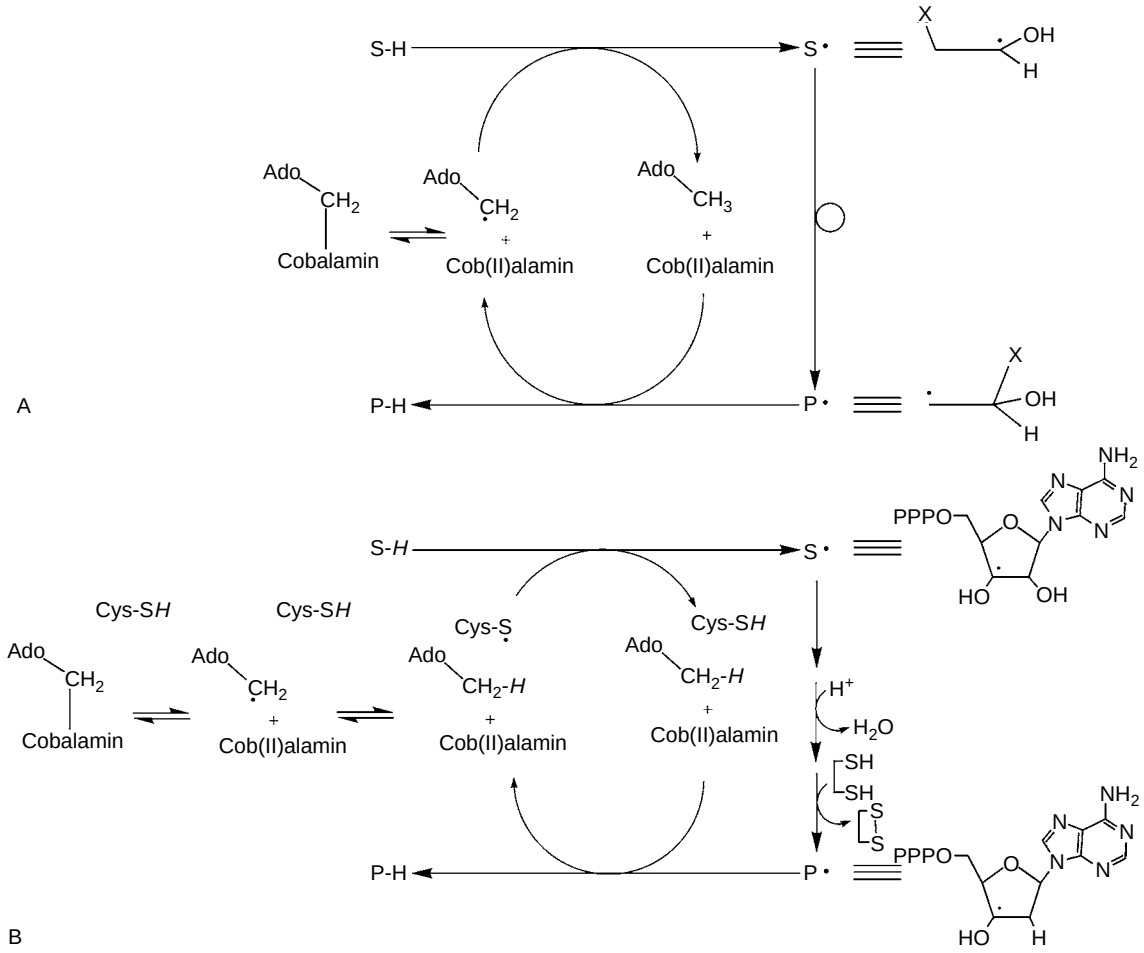



**FIGURE 2** Generalized reaction cycles for enzymes that catalyze radical-mediated rearrangements. (A) The catalytic cycles for enzymes that catalyze rearrangements and eliminations, carbon skeleton rearrangements, and PLP-dependent aminomutase reactions. The structures on the right are examples of the intermediates that would be expected in the conversion of ethanolamine acetaldehyde and ammonia by ethanolamine ammonia-lyase. (B) The catalytic cycle of ribonucleotide triphosphate reductase differs from the cycle in (A) in that the 5′-deoxyadenosyl radical abstracts a hydrogen atom from an active site cysteine thiol to generate a thiyl radical that subsequently generates a substrate-based radical species. The structures on the right show some of the reaction intermediates that form in the course of conversion of ATP to dATP.

adenosylcobalamin. The initial *gem*-diol or *gem*-amino alcohol that is formed in the course of these transformations subsequently eliminates water or ammonia, respectively, to generate the corresponding aldehydes.

Diol and glycerol dehydratases have the best characterized structures in this group. Few sequence similarities exist between the diol and glycerol dehydratases and ethanolamine ammonia-lyase, despite the similarities in the reactions that they catalyze. Nevertheless, members of these groups of enzymes appear to retain coordination of the cobalamin to dimethylbenzimidazole.

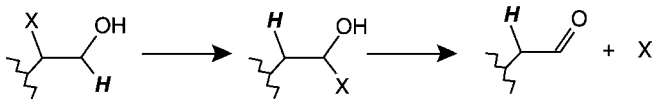
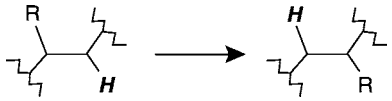
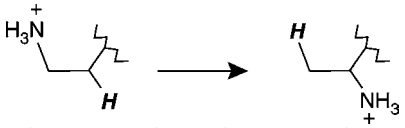
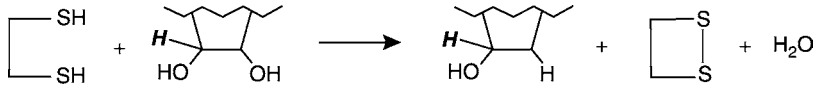
Diol dehydratase and glycerol dehydratase are rapidly inactivated in the course of their catalytic cycle by side reactions that lead the formation of tightly bound inactive cofactor at the active site of these enzymes. The gene clusters that code for the structural genes for these proteins also contain open reading frames whose products have been shown to catalyze the ATP- and $Mg^{2+}$-dependent exchange of inactive cofactor with cofactor from solution. The generality of the reactivation mechanism remains to be determined.

### Carbon Skeleton Rearrangement Reactions

As with the enzymes that catalyze rearrangement and elimination, the enzymes in this group catalyze the interchange of a hydrogen atom on one atom with an alkyl group on the adjacent carbon atom (see Table I). The reactions catalyzed by these enzymes differ from the others in several respects. First, the migrating alkyl group is not eliminated from the product. Second, the mutase reactions are *reversible*. Two significant structural differences have also been noted between these enzymes. First, spectroscopic and structural studies have shown that cobalamin binds in the base-off conformation and that the histidine residue that donates the imidazole ligand is conserved in the members of

**TABLE I**

**Adenosylcobalamin-Dependent Radical-Mediated Transformations**

| | | |
|---|---|---|
| Group 1 | Rearrangement/Elimination reactions |  |
| | Diol dehydratase | 1,2-propanediol → propionaldehyde + water |
| | | ethylene glycol → acetaldehyde + water |
| | Glycerol dehydratase | glycerol → 3-hydroxypropionaldehyde |
| | Ethanolamine ammonialyase | ethanolamine → acetaldehyde + ammonia |
| Group 2 | Carbon skeleton mutases |  |
| | Methylmalonyl-CoA mutase | (2*R*)-methylmalonyl-CoA ⇔ succinyl-CoA |
| | Isobutyryl-CoA mutase | isobutyryl-CoA ⇔ *n*-butyryl-CoA |
| | Glutamate mutase | *S*-glutamate ⇔ (2*S*,3*S*)-3-methylaspartate |
| | 2-Methyleneglutarate mutase | 2-methyleneglutarate ⇔ (*R*)-3-methylitaconate |
| Group 3 | PLP-dependent aminomutases |  |
| | Lysine 5,6-aminomutase | D-lysine ⇔ 2,5-diaminohexanoic acid |
| | | L-β-lysine ⇔ 3,5-diaminohexanoic acid |
| | D-Ornithine 4,5-aminomutase | D-ornithine ⇔ (2*R*,4*S*)-diaminopentanoate |
| Group 4 | Ribonucleotide reduction |  |
| | Ribonucleotide triphosphate reductase | ribonucleotide triphosphate → deoxyribonucleotide triphosphate |

the group. Second, the substrate-based radicals that are formed in the course of the reaction reside ~6Å away from the cob(II)alamin. However, despite these differences, the 5′-deoxyadenosyl radical is involved in generating a substrate-based radical and the catalytic cycle of these enzymes resembles that of the enzymes that catalyze rearrangement and elimination reactions (see Figure 2A).

### PLP-Dependent Aminomutase Reactions

Pyridoxal 5′-phosphate (PLP)-dependent aminomutases catalyze the interchange of a hydrogen atom with an amino group on the adjacent carbon atom. These enzymes (see Table I) have been purified from several strict anaerobes that catabolize lysine or ornithine to organic acids and ammonia. The salient features of the generic aminomutase mechanism are the formation of a Schiff base linkage between the substrate and PLP and the use of 5′-deoxyadenosyl radical to initiate catalysis. In these enzymes, the PLP may assist in the intramolecular migration of the amino group. In addition to the presence of PLP, these enzymes differ from the enzymes that catalyze migration and elimination reactions in that aminomutases catalyze a reversible interchange of a hydrogen atom with the amino group on the adjacent carbon atom and the cofactor binds in a base-off configuration. Despite these differences, the catalytic cycle shown in Figure 2A applies to these enzymes as well.

### Ribonucleotide Triphosphate Reductase Reaction

Ribonucleotide reductases catalyze the conversion of nucleotides to deoxynucleotides in all organisms. The prominent role of these enzymes in nucleotide metabolism has made them attractive targets of antiviral and antitumor therapies. Ribonucleotide reductase can synthesize all four DNA bases from the corresponding nucleosides; however, the activity of the enzymes from all sources is allosterically regulated by the ratio of the deoxyribonucleotides and nucleotides. Four classes

of reductases have been described. Although the overall reactions catalyzed by all of these enzymes are identical, they differ in two respects. First, some use nucleoside diphosphates as substrates, whereas others prefer nucleotide triphosphates. Second, these enzymes differ in the source of the species that initiates the radical turnover cascade. In fact, ribonucleotide reductases have been categorized into four classes based on the free-radical initiators. Only one of these, the class II ribonucleotide triphosphate reductase, is adenosylcobalamin-dependent. However, the catalytic transformations that ensue following the formation of the substrate radical are remarkably similar. The ribonucleotide triphosphate-specific enzyme from *Lactobacillus leichmannii* is the best-characterized example of adenosylcobalamin-dependent reductases.

The mechanistic details of the ribonucleotide reduction differ significantly from the paradigms that have been discussed in the previous sections for the group migration and elimination, aminomutase, and carbon skeleton mutase enzymes. First, ribonucleotide reductases contain a pair of active-site cysteine residues that are oxidized in the course of the reaction and must undergo reduction between each catalytic cycle. Second, a cysteine on the protein forms a thiyl radical that is directly involved in the reaction. Figure 2B shows a general catalytic cycle for the adenosylcobalamin-dependent ribonucleotide reductase, highlighting the differences between this enzyme and other radical-mediated rearrangement reactions. The carbon–cobalt bond of the cofactor is homolyzed by the protein in the presence of allosteric effector; the resulting 5′-deoxyadenosyl radical abstracts a hydrogen atom from an active-site cysteine residue to generate a thiyl radical, which in turn abstracts a hydrogen atom from C3′ of the substrate. In the course of reduction of the substrate to the product, a pair of active-site thiols is oxidized and the active-site thiyl radical is reformed. Reformation of the cofactor and reduction of the active-site thiols prepare the active site for subsequent turnover.

## Methyl Transfer Reactions

Enzymes that catalyze cobalamin-dependent methyl transfer reactions use the potential for heterolytic cleavage of the carbon-cobalt bond of the cofactor (see Figure 1B). The cofactor in these enzymes serves both as a nucleophile and as a leaving group, undergoing transient methylation in the course of the reaction. Although the identities of the donor and acceptor molecules vary among the family of cobalamin-dependent methyl transferase enzymes (Figure 3), the overall catalytic cycles of these enzymes are similar. In this section, we consider methionine synthase as a prototype of the cobalamin-dependent methyltransferases, highlighting the similarities and differences among the members of the methyl transferase family of enzymes.

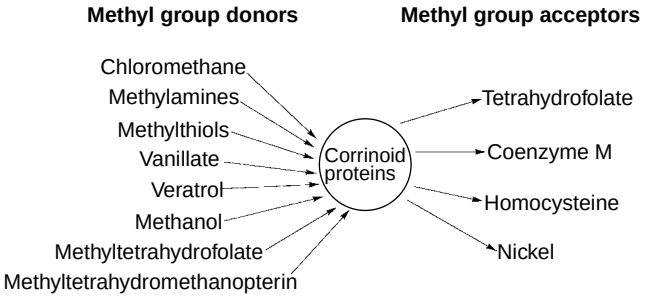



**FIGURE 3** Diversity of methyl group donors and acceptors in cobalamin-dependent methyltransferase enzymes.

### Cobalamin-Dependent Methionine Synthase

Cobalamin-dependent methionine synthase is found in mammals and in *Caenorhabditis elegans*, but not in insects or in plants, which neither produce nor transport cobalamin. It is also found in many prokaryotes, including *Escherichia coli*, but not in Archaea. The enzyme catalyzes the transfer of a methyl group from the tertiary amine methyltetrahydrofolate to the thiol of homocysteine, and the cobalamin cofactor serves as an intermediary in the methyl transfer. A catalytic cycle for methionine synthase is shown in Figure 4.

Methionine synthase is a large monomeric protein (1227 amino acid residues in the enzyme from *E. coli*) containing four discrete modules. The N-terminal module binds and activates homocysteine; the thiol of homocysteine coordinates to a catalytically essential zinc metal ion in this module. The next module in the sequence binds and activates methyltetrahydrofolate. Each of these substrate-binding modules communicates with the third module in the sequence, the $B_{12}$-binding module, to transfer methyl groups to and from the base-off cofactor. The C-terminal module is required for activation of methionine synthase when the cob(I)alamin cofactor becomes oxidized during turnover (~1 in 2000 turnovers results in oxidation *in vitro*). Activation of the inactive cob(II)alamin form of the cofactor requires both a methyl donor and an electron donor. The methyl donor is *S*-adenosyl-L-methionine. In *E. coli* the electron donor is reduced flavodoxin, and in mammals it is methionine synthase reductase, a protein containing a domain with homology to flavodoxin. To allow methyl transfer during reductive activation, the C-terminal domain must also access the cobalamin; a structure of a fragment of methionine synthase in this conformation has recently been determined.

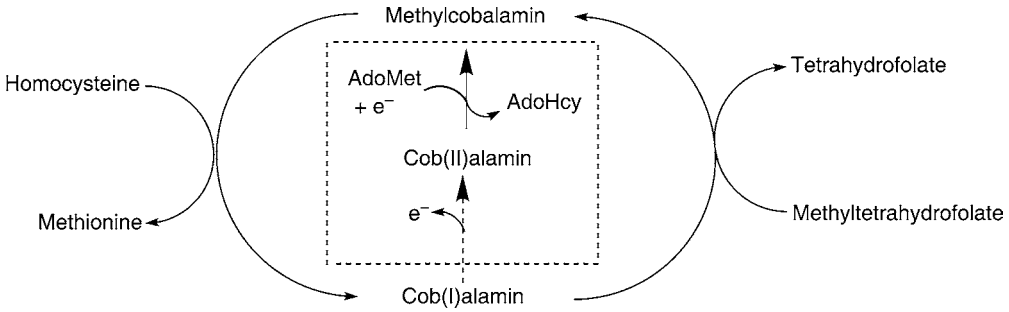



**FIGURE 4** Catalytic cycle of cobalamin-dependent methionine synthase. The dashed box shows the reactivation of the oxidized cofactor by reductive methylation.

## Methyl Transferases Involved in Methanogenesis and Acetogenesis

Although cobalamin-dependent methionine synthase is the only $B_{12}$-dependent methyl transferase in eubacteria and eukaryotes, a large number of enzymes catalyzing similar methyl transfers that employ corrinoid cofactors have been identified in Archaea and acetogenic eubacteria. The diversity in the methyl group donor-acceptor pairs is illustrated in Figure 3. In general, in methanogens, corrinoid-dependent enzymes are involved in the formation of methylcoenzyme M, the methyl thioether that provides the methyl group destined to form methane in methanogenesis. The methyl donors in these reactions include methyltetrahydromethanopterin, which is chemically similar to methyltetrahydrofolate. The methyl group of methyltetrahydromethanopterin is formed by the reduction of carbon dioxide using reducing equivalents derived from molecular hydrogen. Some methanogens can also use simple compounds such as methylamines, methanol, or methylsulfides as the source of methyl groups for methanogenesis. In contrast to cobalamin-dependent methionine synthase, in which a single protein catalyzes methyl transfer from methyltetrahydrofolate to cobalamin and from methylcobalamin to homocysteine, most of these methanogenic methyl transferase reactions require three separate proteins, a corrinoid-containing protein, a methyltransferase that transfers methyl groups from the donor molecule to the corrinoid protein, and a second methyltransferase that transfers methyl groups from the methylcorrinoid protein to the acceptor.

Eubacterial acetogenesis also involves corrinoid-dependent methyl transferases. In these organisms, carbon dioxide and hydrogen are used to generate acetyl CoA. The methyl group of acetyl CoA is generated by reduction of carbon dioxide using reducing equivalents from molecular hydrogen, as in methanogens, and is transferred to a corrinoid cofactor that serves as the direct methyl donor to the enzyme responsible for the synthesis of acetyl CoA from a methyl group and carbon dioxide. Additional acetogenic substrates include vanillate, veratrol, and halogenated aryl or alkene compounds. The precise nature of the chemistry that is promoted by cobalamin with the last substrates remains to be elucidated.

## See Also the Following Articles

DNA Methyltransferases, Bacterial • DNA Methyltransferases: Eubacterial GATC • Pyridoxal Phosphate

## Glossary

**$B_{12}$** A complex organometallic cofactor, also called cobalamin, that is used by enzymes that catalyze group transfer or radical-mediated rearrangement reactions.

**cofactor** A compound that is noncovalently associated with an enzyme and is required for the catalytic activity.

**radical** A species that contains an unpaired electron.

## Further Reading

Banerjee, R. (ed.) (1999). *Chemistry and Biochemistry of $B_{12}$*. John Wiley & Sons, Inc., New York.

Dolphin, D. (ed.) (1982). *$B_{12}$*, Vol. 2, John Wiley & Sons, New York.

Hay, B. P., and Finke, R. G. (1987). Thermolysis of the Co–C bond in adenosylcorrins. 3. Quantification of the axial base effect in adenosylcobalamin by the synthesis and thermolysis of axial base-free adenosylcobinamide: Insights into the energetics of enzyme-assisted cobalt-carbon bond homolysis. *J. Am. Chem. Soc.* **109**, 8012–8018.

Licht, S., and Stubbe, J. (1999). Mechanistic investigations of ribonucleotide reductases. In *Comprehensive Natural Products Chemistry, Enzyme and Enzyme Mechanisms, Proteins, and Aspects of NO Chemistry*, (C. D. Poulter, ed.) Vol. 5, pp. 163–204. Elsevier Science Ltd., Oxford, U.K.

Marsh, E. N., and Drennan, C. L. (2001). Adenosylcobalamin-dependent isomerases: New insights into structure and mechanism. *Curr. Opin. Chem. Biol.* **5**, 499–505.

Sintchak, M. D., Arjara, G., Kellogg, B. A., Stubbe, J., and Drennan, C. L. (2002). The crystal structure of class II ribonucleotide reductase reveals how an allosterically regulated monomer mimics a dimer. *Nat. Struct. Biol.* **9**, 293–300.

Thauer, R. K. (1998). Biochemistry of methanogenesis: A tribute to Marjory Stephenson. 1998 Marjory Stephenson Prize Lecture. *Microbiology* **144**, 2377–2406.

Toraya, T. (2000). The structure and the mechanism of action of coenzyme $B_{12}$-dependent diol dehydratases. *J. Mol. Cat. B* **10**, 87–106.

## Biography

Vahe Bandarian is a postdoctoral fellow at the University of Michigan at Ann Arbor. His research interest is the mechanism of action of adenosylcobalamin-dependent enzymes. He holds a Ph.D. in biochemistry from the University of Wisconsin-Madison and is a recipient of a 2002 Burroughs Wellcome Career Award in Biomedical Sciences.

Rowena G. Matthews is the G. Robert Greenberg Distinguished University Professor of Biological Chemistry and Senior Research Scientist at the Life Sciences Institute of the University of Michigan. Research in her laboratory focuses on the role of vitamins, especially folic acid and vitamin $B_{12}$, in biochemical reactions, and on the regulation of folic acid-dependent metabolism. She received her Ph.D. from the University of Michigan and her B.A. from Radcliffe College, Cambridge, MA. Dr Matthews is a Fellow of the American Association for the Advancement of Science and of the National Academy of Sciences and has served on the Advisory Council of the National Institute of General Medical Sciences.

# Bax and Bcl2 Cell Death Enhancers and Inhibitors

**David L. Vaux**
*The Walter and Eliza Hall Institute, Parkville, Victoria, Australia*

**Apoptosis, the physiological cell death mechanism used by metazoans to remove unwanted cells, is controlled by a family of pro- and anti-apoptotic proteins that bear varying degrees of similarity to a protein called Bcl2. Some members of this family, such as Bcl2 itself, stop cells from activating their suicide mechanism, whereas other members of this family, such as Bax, activate the cell death process.**

## Identification of Bcl2 as a Cell Death Inhibitor

A gene at the site of chromosomal translocations associated with the common lymphoid cancer follicular lymphoma was designated Bcl2 for B-cell leukemia/lymphoma gene number 2. Rather than promoting cell growth and proliferation like most cancer genes, Bcl2 does not stimulate cells to divide but prevents them from activating their endogenous apoptotic cell suicide mechanism. The association of activation of the apoptosis inhibitor Bcl2 with lymphoma was the first evidence that cell death is required to avoid the development of cancer.

## Genetics of Cell Death in *Caenorhabditis elegans*

Although Bcl2 was the first component of the apoptosis mechanism to be recognized at the molecular level in any organism, genetic analysis of programmed cell death in the nematode *C. elegans* revealed that in worms developmental cell death is controlled and implemented by a specific genetic program. Programmed cell death in *C. elegans* fails to occur in *egl-1*, *ced-4*, and *ced-3* loss of function mutants, as well as in *ced-9* gain of function mutants, implying that *egl-1*, *ced-4*, and *ced-3* encode killer proteins and that *ced-9* encodes a survival protein. The expression of human Bcl2 in the worm, and the cloning and sequencing of *ced-9*, showed that they are structurally and functionally homologous. In the worm CED-9 prevents cell death by preventing the adaptor CED-4 from activating the protease CED-3, and in mammalian cells Bcl2 stops apoptosis by indirectly preventing activation of CED-3-like proteases, termed caspases.

## Bcl2 Function

Exactly how Bcl2 prevents apoptosis is unresolved, but there are two main models. In one, Bcl2 acts like CED-9 does in the worm, namely by preventing a mammalian CED-4-like adaptor molecule from activating the caspases. In the other, Bcl2 stops the release of pro-apoptotic proteins from the mitochondria that are sufficient to cause cell death and lead to the further activation of caspases.

## Three Classes of Bcl2 Family Members

Several mammalian proteins have been identified that resemble Bcl2 and bear one or more Bcl2 homology domains, designated BH1-4. These proteins fall into three groups. The first group, to which belong Bcl-x, Bcl-w, and Mcl-1, for example, like Bcl2, is anti-apoptotic and carries three or more of the BH domains. Bax and Bak are members of the second group, which is pro-apoptotic and bears BH domains 1, 2, and 3. The third group of the Bcl2 family is also pro-apoptotic, but carries only the BH3 domain and is therefore often referred to as BH3-only proteins. This class includes proteins such as Bim, Bid, Bad, Bmf, Bik, Hrk, Noxa, and Puma, as well as the *C. elegans* protein EGL-1 (see Figure 1).

## Structure of Bcl2 Family Proteins

Structural studies have shown that Bcl2, Bcl-x, Bcl-w, Bax, and Bid adopt similar three-dimensional folds,

 © 2004, Elsevier Inc. All Rights Reserved. 

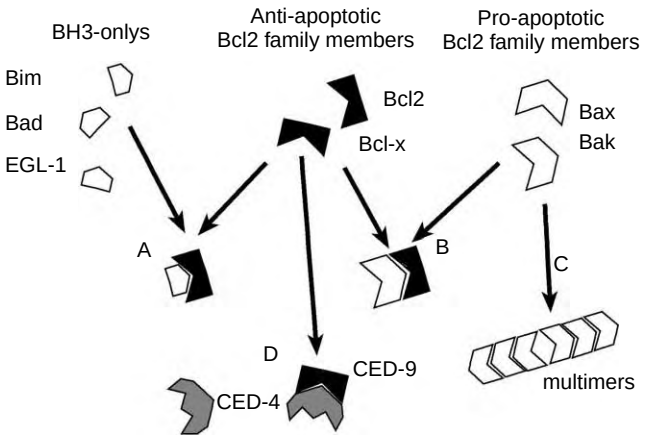



**FIGURE 1** Interactions between Bcl2 family members. (A) Following death signals, BH3-only proteins (e.g., Bim, Bad, Bid, Noxa, Bik, and EGL-1) become activated and can bind strongly to anti-apoptotic family members (e.g., Bcl2, Bcl-x, Bcl-w, and CED-9). (B) In some circumstances anti-apoptotic family members can associate with Bax and Bak. (C) Apoptosis has been associated with movement of Bax to the mitochondria and the formation of multimers of pro-apoptotic Bax and Bak. (D) In *C. elegans*, the anti-apoptotic protein CED-9 can bind to and inhibit the caspase activator CED-4. CED-4 is liberated if enough of the BH3-only protein EGL-1 is present.

composed almost entirely of α-helixes. On the surface is a groove formed from parts of BH1, 2, and 3 that can be occupied either by the carboxy terminus of the protein itself or by the BH3 domain of another Bcl2 family member. Binding of the pro-apoptotic, BH3-only protein EGL-1 via its BH3 domain to the *C. elegans* cell death inhibitor CED-9 blocks its anti-apoptotic activity. In mammals, BH3-only proteins are believed to promote apoptosis by analogous interactions with other mammalian Bcl2 family members. For example, Bak can bind to Bcl-x in an interaction whereby the BH3 domain of Bak binds into the groove on the surface of Bcl-x (Figure 1).

## BH3-Only Family Members

The BH3-only proteins from mammals (Bim, Bad, Bid, Hrk, Bmf, Noxa, Puma, etc.) and *C. elegans* (EGL-1) transduce pro-apoptotic signals to the core cell death machinery by binding, via their BH3 domains, to anti-apoptotic members of the Bcl2 family.

To give a pro-apoptotic signal, the BH3-only proteins can be activated in a wide variety of ways. Some are regulated transcriptionally by proteins such as p53. Others are under posttranslational control. For example, Bid is activated by proteolytic cleavage, Bad is activated by dephosphorylation, and Bim and Bmf are activated when released from sequestration by components of the cell's cytoskeleton. In this way, pro-apoptotic signals from various parts of the cell can be integrated into a common cell death effector mechanism.

## Bax and Bak

Bax and Bak are pro-apoptotic proteins that bear BH1-3 domains. The deletion of genes for either Bax or Bak results in a very mild phenotype in mice, but the deletion of both has very dramatic effects, suggesting that there is a requirement for Bax or Bak for cell death in many circumstances but that the presence of one can compensate for absence of the other. Most mice lacking both Bax and Bak die perinatally. Those that do survive have persistent interdigital webs, imperforate vaginas, and accumulate excess neurons and blood cells. The fact that lymphocytes from these animals retain full sensitivity to cell death stimulated by the ligation of the tumor necrosis factor (TNF) receptor family member Fas shows that in these cells apoptosis can be activated by two independent pathways, only one of which is regulated by Bcl2 family members.

How Bax and Bak function is not certain. Cells lacking Bax and Bak are resistant to apoptosis caused by the overexpression of BH3-only proteins, and BH3-only proteins have been reported to bind to Bax and Bak, but the interactions appear to be weak and may not occur *in vivo*. Apoptosis is associated with the translocation of Bax from the cytosol to the mitochondria and the formation of multimers of Bax and Bak that can be detected by cross-linking (Figure 1). Some believe that these multimers can act as channels in the mitochondrial membranes allowing release of proteins such as cytochrome c and Smac/Diablo.

## Role of the Mitochondria

In almost all cases, apoptosis is associated with the release of proteins such as cytochrome c and Smac/Diablo from the mitochondria. It has not yet been resolved whether this is necessary or sufficient for cell death. Some groups believe that Bcl2 prevents apoptosis by stopping the release of these mitochondrial proteins, whereas Bax and Bak cause apoptosis by enabling their release. Based on comparisons of their 3D structure with that of diphtheria toxin, it has been proposed that Bcl2, Bcl-xl, Bax, and Bak form or regulate channels in the mitochondrial outer membrane through which the proteins leave the mitochondria. The BH3-only proteins somehow cause Bax and Bak to form these channels. According to this model, cells subsequently die either because of the loss of mitochondrial function or because cytochrome c activates the CED-4 homologue Apaf-1, which in turn activates the caspases.

Other groups believe that Bcl2 inhibits, whereas Bax promotes, the activation of caspases that are sufficient to cause apoptosis independently of the mitochondria. According to this model, these caspases also cause

secondary damage to the mitochondria, resulting in the release of proteins such as cytochrome c and Smac/Diablo that accelerate the death process.

## Roles of Bcl2 Family Members *in vivo*

To determine their essential functions, many of the genes for Bcl2 family members have been deleted in mice. The deletion of Bcl-x results in death in early embryogenesis. Mice lacking Bcl2 genes are healthy at birth but grow poorly, turn gray, and usually die of renal failure caused by cysts in their kidneys. White blood cells from these mice are very sensitive to apoptotic stimuli.

Although mice lacking Bax or Bak have no major abnormalities, mice lacking both these pro-apoptotic proteins accumulate large numbers of white blood cells and have extra neurons. Cells from these mice are resistant to apoptosis triggered by BH3-only proteins, but not that triggered by members of the TNF family of receptors.

Mice lacking genes for Bim have excess white blood cells, and these are resistant to some cell-death-inducing drugs but not others. The fact that mice lacking genes for both Bim and Bcl2 have different phenotypes from those missing one or the other indicates that, although Bim can be inhibited by Bcl2, it can also act independently of Bcl2 and, conversely, although Bcl2 can be inhibited by Bim, it can also act independently of Bim.

## Therapeutic Implications

The association of Bcl2 with follicular lymphoma and the ability of antiapoptotic Bcl2 family members to inhibit chemotherapy-induced apoptosis have led to speculation that antagonizing anti-apoptotic Bcl2 family members or reducing their levels will promote the death of cancer cells. Consequently, trials are underway to use antisense against Bcl2 and to test novel drugs that mimic BH3-only proteins against a variety of cancers.

### See Also the Following Articles

Caspases and Cell Death • Cell Death by Apoptosis and Necrosis • Cytochrome *c*

### Glossary

**apoptosis** A physiological form of cell death with a characteristic morphology whose mechanism is shared by metazoans.

**Bcl2 family proteins** Proteins that have a structural similarity to the apoptosis inhibitory protein Bcl2.

**BH domains** Bcl2 homology domains; there are four, BH1-4.

**BH3-only proteins** A group of pro-apoptotic proteins that have the BH3 domain but no other BH domains.

**caspases** Cysteine proteases with aspartic acid specificity that are related to the *C. elegans* caspase CED-3 that is essential for programmed cell death in the worm.

***ced* genes** Genes that implement programmed cell death in the worm *C. elegans*.

### Further Reading

Adams, J. M., and Cory, S. (2001). Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem. Sci.* **26**, 61–66.

Huang, D. C. S., and Strasser, A. (2000). BH3-only proteins – essential initiators of apoptotic cell death. *Cell* **103**, 839–842.

Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., Ma, A., Golden, J. A., Evan, G., Korsmeyer, S. J., MacGregor, G. R., Thompson, C. B. (2000). The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. *Mol. Cell* **6**, 1389–1399.

Metzstein, M. M., Stanfield, G. M., and Horvitz, H. R. (1998). Genetics of programmed cell death in *C. elegans* – past, present and future. *Trends Genetics* **14**, 410–416.

Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. (1997). Structure of bcl-x(l)-bak peptide complex – recognition between regulators of apoptosis. *Science* **275**, 983–986.

Vaux, D. L., Cory, S., and Adams, J. M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**, 440–442.

Vaux, D. L., Weissman, I. L., and Kim, S. K. (1992). Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. *Science* **258**, 1955–1957.

### Biography

David L. Vaux is a Laboratory Head at the Walter and Eliza Hall Institute in Melbourne, Australia. His principal research interest is the molecular biology of apoptosis. He holds an M.D. and a Ph.D. from Melbourne University and received postdoctoral training at Stanford University. He was the first to recognize that Bcl2 was an inhibitor of cell death, and he linked apoptosis in mammals to programmed cell death in the worm.

# B-Cell Antigen Receptor

**Thomas M. Yankee and Edward A. Clark**
*University of Washington, Seattle, Washington, USA*

**B-cell functions in the immune system are dependent on their ability to recognize foreign antigens and to discriminate between foreign antigens and self-antigens. The complex of proteins responsible for the identification of foreign antigens is the B-cell antigen receptor (BCR) complex. Signals originating with the BCR can lead to a variety of cell fates, depending on the developmental stage of the B-cell and the concentration and avidity of the antigen. B-cell precursors in the bone marrow require BCR signals for survival. Once a competent signaling complex is formed, the BCR is tested to ensure that it does not react with self-antigens. If the BCR is ligated by self-antigens, it must either change its specificity or undergo cell death. Later in B-cell development, basal BCR signaling is required for the survival and homeostatic maintenance of the B-cell pool. BCR stimulation of mature B-cells initiates an immune response, characterized by the proliferation and differentiation of B-cells into either antibody-producing plasma cells or memory B-cells. Hence, a competent BCR that recognizes foreign antigens and not self-antigens is critical for the development and maintenance of B-cells and for the initiation of humoral immune responses.**

## Structure of the B-Cell Antigen Receptor

The BCR complex contains a membrane-bound immunoglobulin (mIg). mIg can be one of five isotypes: IgM, IgD, IgA, IgG, or IgE. The isotype of mIg expressed on a given B-cell varies with the stage of development and activation. Immature and transitional B-cells express mIgM, whereas mature resting B-cells express mostly mIgD and some mIgM. Cells that have been activated undergo a process called isotype class-switching and may then express mIgG, mIgA, or mIgE in addition to soluble IgG, IgA, or IgE. The process of isotype class-switching results in the activation of other hematopoietic cells. Each molecule of mIgM contains two heavy-chain (H) and two light-chain (L) proteins, that is $H_2L_2$ (Figure 1). Disulfide bonds link the heavy chains to one another and each heavy chain is disulfide-bonded to a light chain. At the membrane proximal end of the heavy chain is a transmembrane domain followed by a short intracellular domain. mIgM and mIgD contain only three intracellular residues that anchor the protein in the membrane. Other classes of mIg contain slightly longer intracellular domains and appear to mediate functions only present in B-cells that express mIgG, mIgA, or mIgE.

The short intracellular domains of mIgM and mIgD alone cannot generate a signal; mIg-associated accessory proteins fulfill this function. There are two such accessory molecules, Ig$\alpha$ (also called CD79a) and Ig$\beta$ (CD79b). The interaction between mIg and Ig$\alpha$/Ig$\beta$ depends on the transmembrane domains of each protein. The transmembrane domains of mIg, Ig$\alpha$, and Ig$\beta$ are $\alpha$-helices and contain both polar and nonpolar regions. The polar regions are critical for protein–protein interactions among the BCR components. mIg contains polar residues on both sides of the $\alpha$-helix. One side is conserved among all Ig isotypes and the other side is isotype specific. The residues that are isotype specific allow for oligomerization of the BCR complex. The degree of oligomerization varies with each Ig isotype. The side of the molecule that is conserved among all isotypes participates in the association between mIg and Ig$\alpha$/Ig$\beta$.

Like mIg, the transmembrane domain of Ig$\alpha$ contains polar residues on both sides of the $\alpha$-helix. In contrast, Ig$\beta$ contains polar residues on only one side of the $\alpha$-helix. Thus, a favored model for the structure of the BCR complex is that Ig$\alpha$ bridges mIg and Ig$\beta$ (Figure 1). According to this model, when the BCR complex is assembled, the polar residues within the transmembrane domains are masked by protein–protein interactions. This is essential for the surface expression and stability of the BCR.

## B-Cell Antigen Receptor Diversity

In order to contribute to protective immunity to a range of pathogens, B-cells must be able to recognize a vast array of antigens. The diversity of the Ig repertoire is accomplished using three factors: combinatorial diversity, junctional diversity, and somatic hypermutation. Combinatorial diversity occurs through the

 © 2004, Elsevier Inc. All Rights Reserved. 

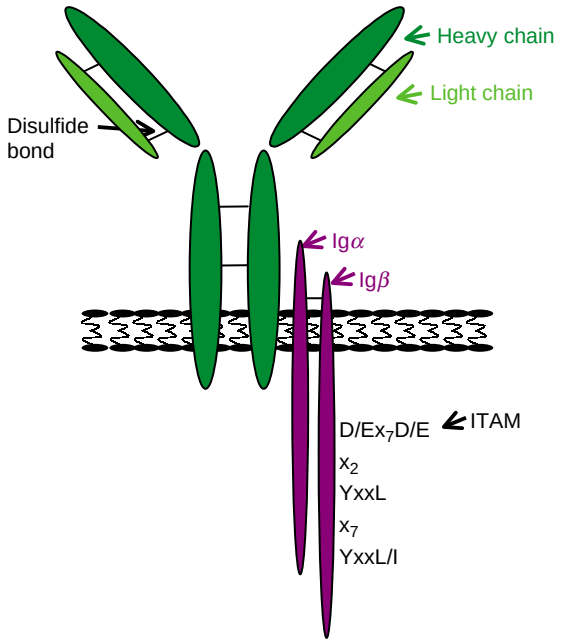



**FIGURE 1** The structure of the BCR. The BCR consists of two heavy chains disulfide-bonded to one another. Each heavy chain is disulfide-bonded to a light chain. Ig$\alpha$ and Ig$\beta$ are also disulfide-linked. mIg and Ig$\alpha$/Ig$\beta$ are noncovalently coupled. Also shown is the ITAM motif on Ig$\beta$; Ig$\alpha$ and Ig$\beta$ each have one ITAM motif.

recombination of sets of heavy- and light-chain gene segments. Three gene segments, variable ($V_H$), diversity ($D_H$), and joining ($J_H$), recombine to form the variable region of the heavy chain. There are many $V_H$ gene segments, $D_H$ gene segments, and $J_H$ gene segments. Thus, sets of different segments recombine to form a range of different $VDJ_H$ genes. The light chain undergoes a similar process of recombination. To generate the light chain, two gene loci, $\kappa$ and $\lambda$, undergo recombination. Here, $V_L$ and $J_L$ gene segments recombine and result in $VJ_L$. The combination of heavy-chain rearrangement and light-chain rearrangement results in substantial BCR diversity.

Junctional diversity also arises from the recombination of the gene segments. When a $V_H$ gene segment recombines, nucleotides may be added or subtracted. This results in the addition or loss of amino acids or a shift in the reading frame of the new gene. The third mechanism by which diversity is introduced to the BCR repertoire is through somatic hypermutation (discussed later).

## B-Cell Antigen Receptor Signaling

Ligation of the BCR complex triggers a series of events that ultimately affects the fate of the cell. A B-cell stimulated through the antigen receptor may proliferate, differentiate, or undergo apoptosis. B-cells are also very efficient antigen-presenting cells. BCR signaling is essential for antigen internalization and processing after the BCR binds and captures antigen.

### Antibody–Antigen Interactions

Antigens interact with the variable regions of mIg. Within the variable region, there are framework regions interspersed with three hypervariable regions, the areas that contain the most variability. The framework regions, folded as $\beta$-sheets, provide much of the antibody structure. The hypervariable regions are located on one edge of the $\beta$-sheets. To provide even greater repertoire diversity, the hypervariable regions of the heavy and light chain are within close proximity to one another and together create the antigen-binding site. Haptens and other small antigens bind the antibody in the grooves between the hypervariable regions of the heavy and light chains. Interactions between the BCR and larger antigens may extend into the framework region. The interactions between the antibodies and antigens are noncovalent in nature. These interactions may consist of electrostatic interactions, van der Waals interactions, hydrophobic interactions, and hydrogen bonding.

### Early Signaling Events

Ig$\alpha$ and Ig$\beta$ each contains tyrosine residues that are phosphorylated following BCR engagement. Two of the tyrosine phosphorylation sites on Ig$\alpha$ and Ig$\beta$ are located within immunoreceptor tyrosine-based activated motifs (ITAMs) (Figure 1). These regions contain six conserved residues in the specific configuration, D/ExxxxxxxD/ExxYxxLxxxxxxxYxxL/I. The spacing between the tyrosine residues in the ITAM results in these residues being positioned on the same side of an $\alpha$-helix, facilitating the interactions with downstream SH2-domain-containing signaling molecules. (SH2 domains are motifs that bind phosphotyrosine and the surrounding residues.)

In addition to the ITAM tyrosine residues, Ig$\alpha$ and Ig$\beta$ contain other residues that become phosphorylated. Non-ITAM tyrosine residues may also recruit SH2-domain-containing signaling proteins. Serine/threonine phosphorylation of Ig$\alpha$ may negatively regulate phosphorylation of the ITAM tyrosine residues and, therefore, may negatively regulate downstream signaling.

Three families of protein tyrosine kinases (PTKs) play key roles in the initiation of BCR signaling (Figure 2). These kinases are Src-family PTKs, such as Lyn; the Syk/ZAP-70-family PTKs, such as Syk; and the Tec-family PTKs, such as Btk. Following BCR ligation, Lyn becomes activated and phosphorylates the ITAM tyrosines. The phosphorylated ITAM becomes the docking sites for Syk, which has two tandem SH2 domains.

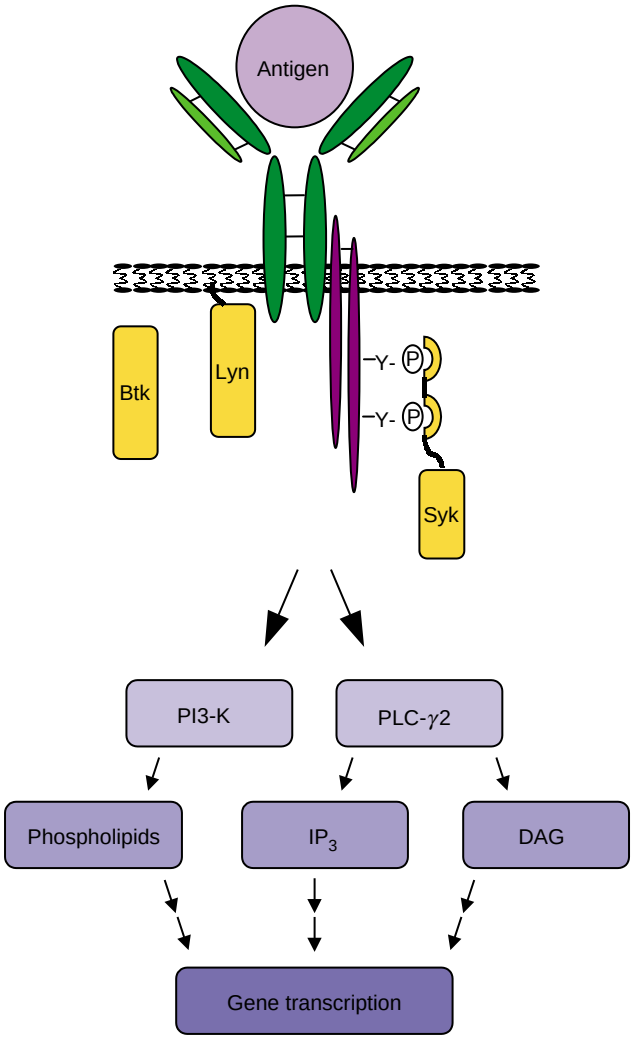



**FIGURE 2** BCR-mediated signaling events. Following antigen binding to the BCR complex, tyrosine residues on Ig$\alpha$ and Ig$\beta$ become phosphorylated and bound by the tandem SH2 domains of the Syk. Lyn is also activated. The activation of these two PTKs trigger many downstream signaling cascades, including the activation of PI3-K and PLC-$\gamma$2.

The binding of the SH2 domains of Syk to the phospho-ITAM contributes to its activation. Syk can also become activated in the absence of Src-family PTKs. A small amount of Syk is constitutively associated with the BCR complex. Cross-linking the BCR leads to the clustering of Syk, which then may transautophosphorylate and become activated. Activation of Syk and Lyn, then, leads to the activation of Btk. The combination of Syk, Lyn, and Btk activity is necessary for optimal signaling through the BCR.

### Downstream Signaling Events

The activation of Syk-, Src-, and Tec-family PTKs trigger a number of downstream signaling pathways (Figure 2). For example, phosphatidylinositol 3′-kinase (PI3-K) and phospholipase C-$\gamma$2 (PLC-$\gamma$2) are two enzymes that generate second messengers in BCR signaling. PI3-K is a lipid kinase important for the recruitment and activation of PH-domain-containing proteins. (PH domains are motifs that bind phospholipids.) PLC-$\gamma$2 generates inositol-1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG), second messengers critical for calcium influx and protein kinase C activation. The activation of these signaling pathways, along with many other pathways, leads to gene transcription and cell-fate decisions.

In addition to gene transcription, BCR signaling is also critical for antigen internalization and processing. When the BCR binds an antigen, the BCR complex is internalized. The antigen is then processed and presented on the cell surface in order to activate antigen-specific T cells.

## The B-Cell Antigen Receptor and the Immune Response

When antigen and other immune cells activate a B-cell, a complex series of events takes place that can result in proliferation, somatic hypermutation, isotype class-switching, and differentiation. The BCR on mature naïve B-cells may be able to recognize more than one antigen. This multispecific nature of the BCR results in relatively low affinity to any specific antigen. Somatic hypermutation is a process by which high-affinity BCRs are generated. Under some conditions following BCR ligation, a somatic hypermutation program is induced. During somatic hypermutation mutations are introduced into the V segment of the heavy- and light-chain genes. After somatic hypermutation, B-cells expressing BCRs with high affinity are selected for further expansion. In this way, the immune system creates B-cells that have produced high affinity antibodies tailor-made for specific pathogens.

The other major changes in the BCR following B-cell activation include isotype class-switching. The genomic organization of mIg is the recombined variable region followed by the constant genes of $\mu$, $\partial$, $\gamma$3, $\gamma$1, $\gamma$2b, $\gamma$2a, $\varepsilon$, and $\alpha$ in mice and $\mu$, $\partial$, $\gamma$3, $\gamma$1 $\alpha$1, $\gamma$2, $\gamma$4, $\varepsilon$, and $\alpha$2 in humans. Following the activation of the B-cell through certain key receptors such as CD40, DNA recombination can occur so that the $V_H$ joins a $C_H$ segment of another isotype. This process is a guided repetitive DNA sequence preceding each constant domain known as a switch region. The precise mechanism of class-switching is unknown, but probably involves DNA recombination between homologous repeats in the switch regions of different constant genes.

The result of somatic hypermutation and class-switching is the generation of highly specific antibodies of an isotype that generates an effective immune response.

In particular, the constant regions of the antibody bind specific receptors on other immune cells. For example, the constant region of IgG$\gamma$1 and IgG$\gamma$3 bind Fc$\gamma$ receptors on macrophages and neutrophils leading to phagocytosis of the antigen, whereas the constant region of IgE binds Fc$\varepsilon$ receptors on mast cells and basophils leading to the secretion of inflammatory agents.

Membrane-bound forms of IgG, IgA, and IgE may function differently than mIgM and mIgD. Cross-linking mIgG leads to the proliferation and antibody secretion of mIgG-expressing B-cells. Although mIgG and mIgM activate similar downstream signaling pathways, mIgG cross-linking triggers a much more robust response than does mIgM cross-linking. This may be due to the fact that mIgG is resistant to down-regulation by certain B-cell co-receptors, such as CD22. The mechanism for this resistance may be related to differences in the intracellular domains between mIgM and mIgG.

## Summary

The BCR complex is a critical component of the humoral immune response. The BCR repertoire must be diverse enough to recognize an incredible number of foreign antigens, but must also avoid targeting self-antigens. Ligation of the BCR triggers a complex series of events that include the activation of PTKs, the phosphorylation of Ig$\alpha$ and Ig$\beta$, and the initiation of multiple signaling cascades. These signaling pathways lead to cell-fate decisions, antigen presentation, and an immune response.

## See Also the Following Articles

Diacylglycerol Kinases and Phosphatidic Acid Phosphatases • Inositol Phosphate Kinases and Phosphatases • Phosphatidylinositol Bisphosphate and Trisphosphate • Protein Tyrosine Phosphatases • Src Family of Protein Tyrosine Kinases

## Glossary

**antibody** A protein that is produced in response to immune challenge and binds specifically to a particular antigen.

**antigen** A molecule that specifically binds an antibody.

**avidity** The sum of the binding strengths of all points of interaction between an antigen and an immunoglobulin.

**humoral immune response** The antibody-mediated response to a specific antigen.

**immunoglobulin** A protein complex with a characteristic structure of heavy and light chains.

**isotype** A class of immunoglobulin as determined by the constant region.

**somatic hypermutation** The process by which mutations are introduced into the heavy- and light-chain genes.

## Further Reading

Cyster, J. (1997). Signaling thresholds and interclonal competition in preimmune B-cell selection. *Immunol. Rev.* **156**, 87–101.

Janeway, C., Travers, P., Walport, M., and Schlomchick, M. (2001). *Immunobiology: The Immune System in Health and Disease*. Garland Publishing, New York.

Matsuuchi, L., and Gold, M. (2001). New views on BCR structure and function. *Curr. Opinion Immunol.* **13**, 270–277.

Melchers, F., ten Boekel, E., Seidl, T., Kong, X., Yamagami, T., Onishi, K., Shimizu, T., Rolink, A., and Andersson, J. (2000). Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of b-cell development from immature to mature B-cells. *Immunol. Rev.* **175**, 33–46.

Niiro, H., and Clark, E. (2002). Regulation of B-cell fate by antigen receptor signals. *Nat. Rev. Immunol.* **2**, 945–956.

Reth, M. (1992). Antigen receptors on B-lymphocytes. *Annu. Rev. Immunol.* **10**, 97–121.

Yankee, T., and Clark, E. (2000). Signaling through the B-cell antigen receptor in developing B-cells. *Rev. Immunogenet.* **2**, 185–203.

## Biography

Dr. Thomas M. Yankee is a Senior Fellow in the Department of Microbiology at the University of Washington, Seattle. His principal interest is the regulation of cell fate in B- and T-lymphocytes. He holds a Pharm.D. degree from the University of Illinois and a Ph.D. degree from Purdue University.

Dr. Edward A. Clark is a Professor of Microbiology and Immunology at the University of Washington, Seattle. His principal interest is the regulation of B-cells and dendritic cells during immune responses. He holds a Ph.D. degree from the University of California, Los Angeles. He has helped to discover and characterize a number of receptors and signaling molecules in B-lymphocytes such as CD40, CD80, and Syk.

# Bile Salts and their Metabolism

**Ulrich Beuers and Thomas Pusl**
*University of Munich, Munich, Germany*

**Bile salts originate from conversion of cholesterol in the liver, a major pathway for elimination of cholesterol from the body. Bile salts together with phospholipids and cholesterol are the major organic solutes in bile and are the key driving force of bile formation being actively secreted into bile canaliculi across the apical membranes of hepatocytes. Bile salts undergo an efficient enterohepatic circulation. Their structure is amphipathic with a hydrophobic and hydrophilic side allowing them to interact with both lipids and the aqueous environment. Molecular self-aggregation occurs, by means of micelle formation, above a critical micellar concentration. Bile salt micelles can solubilize other lipophilic molecules such as cholesterol, phospholipids, and monoglycerides to form mixed micelles, acting as carriers for these lipids in bile and in the intestine. Such micelle formation promotes absorption of dietary lipids and fat-soluble vitamins in the small intestine. Bile salts may become cytotoxic when their intracellular concentrations increase beyond physiological levels due to impairment of bile secretion as observed in a variety of pathological conditions, particularly cholestatic liver diseases. Therefore, bile salt homeostasis is tightly regulated. Bile salts have recently been shown to represent potent intracellular signaling molecules that activate nuclear receptors and modulate cytosolic signaling cascades, thereby regulating their own metabolism and transport.**

## Bile Salt Synthesis and its Regulation

Bile salts consist of a steroid nucleus with its hydroxyl or other substituents and an aliphatic side chain of variable length. They are synthesized in pericentral hepatocytes from cholesterol by different pathways, which involve up to 17 enzymes located in the endoplasmatic reticulum, mitochondria, cytosol, and peroxisomes (Figure 1). The rate-limiting step of the classic bile salt biosynthetic pathway, also known as the "neutral" pathway, is 7$\alpha$-hydroxylation of cholesterol by a microsomal cytochrome P-450 monooxygenase (CYP7A1). End products of the complex biosynthetic steps are the two major human primary bile salts, cholate (C; 3$\alpha$,7$\alpha$,12$\alpha$-trihydroxy-5$\beta$-cholanoate) and chenodeoxycholate (CDC; 3$\alpha$,7$\alpha$-dihydroxy-5$\beta$-cholanoate). These primary bile salts undergo further modifications during enterohepatic cycling by bacterial enzymes in the distal intestine which lead to dehydroxylation at C-7 of C or CDC to form the secondary bile salts, deoxycholate (DC; 3$\alpha$,12$\alpha$-dihydroxy-5$\beta$-cholanoate) and lithocholate (LC; 3$\alpha$-hydroxy-5$\beta$-cholanoate), respectively, or to epimerization of the hydroxy group at C-7 of CDC to form ursodeoxycholate (UDC; 3$\alpha$,7$\beta$-dihydroxy-5$\beta$-cholanoate). A second pathway (commonly referred to as the "alternative" or "acidic" pathway) is initiated by the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1), which allows for the conversion of cholesterol to both 27-hydroxycholesterol and 3$\beta$-hydroxy-5-cholestenoic acid. Although CYP27A1 is expressed also in peripheral tissues such as macrophages and vascular endothelium, the liver remains the sole site for complete bile salt formation. The acidic pathway provides a mechanism by which excess oxidized cholesterol generated in peripheral tissues can be removed through conversion to bile salts. The relative contribution of CYP7A1 and CYP27A1 to overall bile salt synthesis is unclear, but CYP7A1 has been estimated to account for 75% (mice) to 90–95% (human) of total bile salt synthesis. Cholesterol 25-hydroxylase and cholesterol 24-hydroxylase (CYP46A1) may also initiate bile salt synthesis *in vitro*, but their contribution to bile salt formation *in vivo* may be limited.

Bile salt synthesis is highly regulated and subject to feedforward and feedback control whereby bile salts down-regulate their own synthesis and oxysterols up-regulate bile salt synthesis, mainly by regulating CYP7A1 gene transcription (Figure 2). Promoter analyses of the CYP7A1 gene have identified two bile acid response elements, which are highly conserved among different species, contain hexameric repeats of an AGGTCA sequence, and are binding sites for nuclear hormone receptors. Nuclear hormone receptors are ligand-activated transcription factors with a highly conserved DNA-binding domain (DBD) in the N-terminal region and a moderately conserved ligand-binding domain (LBD) in the C-terminal region. Upon ligand binding to the LBD, nuclear receptors undergo conformational changes that allow dissociation of corepressors and recruitment of coactivator proteins to

 © 2004, Elsevier Inc. All Rights Reserved. 

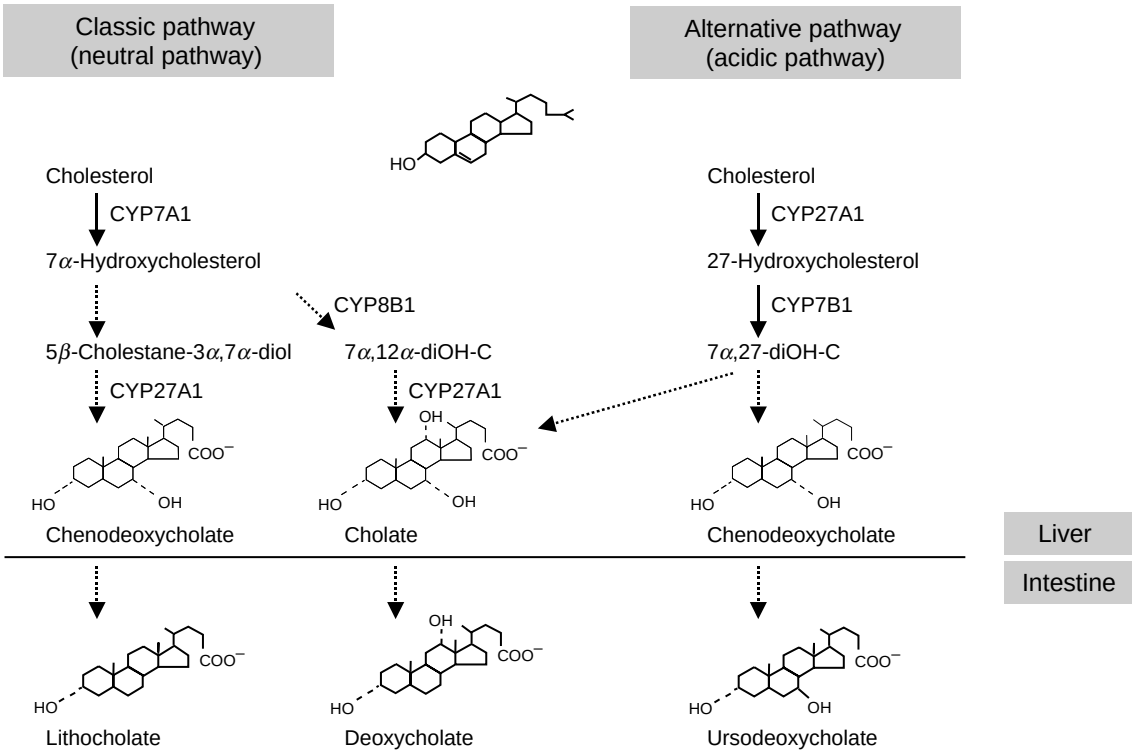



**FIGURE 1** Major bile salt biosynthetic pathways in the liver. The initial and rate-determining step of the classic ("neutral") pathway is the conversion of cholesterol to 7$\alpha$-hydroxycholesterol by microsomal cholesterol 7$\alpha$-hydroxylase (CYP7A1) leading to formation of cholate and chenodeoxycholate. The initial step of the alternative ("acidic") pathway is the conversion of cholesterol to 27-hydroxycholesterol by mitochondrial sterol 27-hydroxylase (CYP27A1) leading to formation mainly of chenodeoxycholate. These primary bile salts undergo further structural modifications by bacterial enzymes during enterohepatic cycling resulting in the formation of the secondary and tertiary bile salts, deoxycholate, lithocholate, and ursodeoxycholate. Only major biosynthetic steps are shown. CYP7B1, oxysterol 7$\alpha$-hydroxylase; CYP8B1, sterol 12$\alpha$-hydroxylase; 7$\alpha$,27-diOH-C, 7$\alpha$,27-dihydroxycholesterol; 7$\alpha$,12$\alpha$-diOH-C, 7$\alpha$,12$\alpha$-dihydroxy-4-cholestene-3-one.

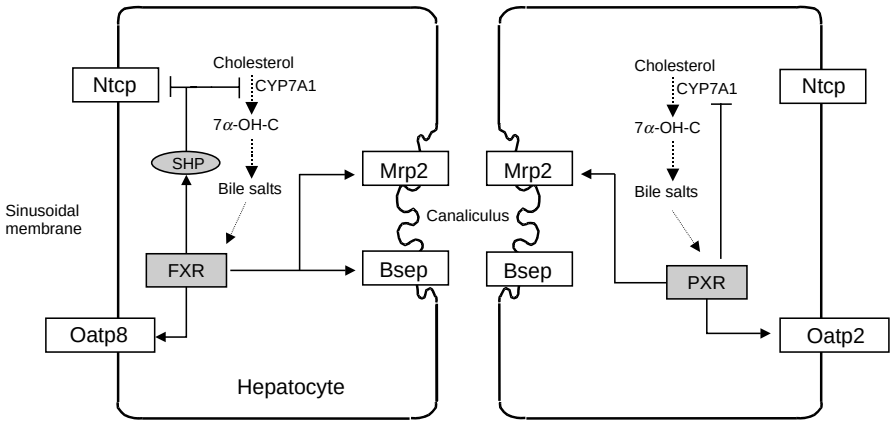



**FIGURE 2** Regulation of bile salt transport and metabolism by nuclear bile salt receptors in hepatocytes. The transport of bile salts is vectorial in liver. Bile salts are taken up by the Ntcp and Oatp in the sinusoidal membrane of hepatocytes and are excreted into bile via the Bsep and the conjugate export pump (Mrp2) located in the canalicular membrane. The FXR acts as a bile salt sensor and represses the transcription of cholesterol 7$\alpha$-hydroxylase (CYP7A1) and Ntcp genes via up-regulation of small heterodimer partner-1 (SHP-1), thereby inhibiting bile salt synthesis and uptake. FXR also stimulates expression of Bsep, Mrp2, and Oatp8. PXR represses bile salt synthesis by down-regulation of transcription of CYP7A1. PXR also stimulates the expression of Mrp2 and Oatp2. 7$\alpha$-OH-C, 7$\alpha$-hydroxycholesterol ($\rightarrow$ activation; $\dashv$ repression).

activate the expression of target genes. The nuclear hormone receptor superfamily includes receptors for steroid and thyroid hormones, retinoids and vitamin A and D, as well as different so-called orphan receptors, whose ligands were initially unknown. Analyses of orphan receptor expression patterns in enterohepatic tissue have identified bile salts, including CDC and C, as the endogenous ligands of farnesoid X receptor (FXR). In the liver, bile salt activation of FXR represses CYP7A1 transcription by an indirect mechanism involving nuclear receptors small heterodimer partner (SHP) and liver receptor homologue 1 (LRH1). Activation of pregnane X receptor (PXR), a promiscuous bile salt receptor, also results in repression of CYP7A1. The liver X receptor $\alpha$ (LXR$\alpha$), abundantly expressed in the liver and bound by oxysterol ligands, has been shown to mediate the feedforward cholesterol catabolism to bile acids by up-regulation of CYP7A1. Nuclear hormone receptor-independent pathways may also be involved in bile salt feedback inhibition of gene transcription based on protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signaling pathways as well as bile-salt-induced release of inflammatory cytokines.

## Bile Salt Transport and its Regulation

After their biosynthesis and conjugation mainly to glycine and taurine in hepatocytes, bile salts are secreted into bile and reach the intestinal lumen. More than 95% of intestinal conjugated bile salts are actively reabsorbed mainly from the distal ileum by an apical sodium-dependent bile salt transporter (Asbt) and reach the liver via the portal vein, the majority being bound to albumin. Hepatic extraction followed by resecretion into bile completes the enterohepatic circulation of bile salts. In humans, the normal bile salt pool size averages 3–4 g recirculating 5–10 times each day. The hepatocyte plays a key role in the vectorial transport of bile salts from portal vein to the bile canaliculus, with distinct bile salt transport systems at its basolateral (sinusoidal) and apical (canalicular) plasma membranes (Figure 2). Functional impairment of bile salt transport at any level of the hepatocyte may be an important cause of cholestasis, a syndrome characterized by a reduction in bile flow and retention of biliary constituents in serum, liver, and other organs leading to biochemical, morphological, and clinical alterations.

Hepatocellular uptake of bile salts at the basolateral membrane is the first step in hepatic bile salt transport, and is predominantly mediated by the $Na^+$-taurocholate-cotransporting polypeptide (Ntcp) and to a lesser extent by the organic anion transporting protein (Oatp) family. The 362-amino acid Ntcp with a molecular mass of 50 kDa has been cloned from human liver and mediates secondary active transport of bile salts, using an inwardly directed sodium gradient generated by $Na^+$-$K^+$-ATPase and the intracellular negative electrical potential generated in part by a potassium channel. Members of the Oatp family mediate sodium-independent uptake of organic anions and mainly unconjugated bile salts. Tissue distribution of Oatps is not restricted to the liver, where they are exclusively located in the basolateral membrane of the hepatocyte.

Little is known about the intrahepatocellular transport of bile salts. Cytosolic proteins such as a 36 kDa bile salt binding protein in human liver are considered to play a major role in the intracellular trafficking of bile salts, but rapid diffusion may also contribute to bile salt trafficking across the cell. In addition, bile salt partitioning into organelles may be observed when the intracellular bile salt load is increasing.

Under physiologic conditions, bile salt levels in the hepatocyte remain low, indicating the existence of efficient secretory mechanisms. Canalicular excretion of bile salts is predominantly achieved by the ATP-dependent bile salt export pump (Bsep), which transports monovalent bile salts against a steep gradient (up to 1000-fold) across the apical liver cell membrane into the canaliculus. This 60 kDa protein is a member of a large family of ATP-binding cassette (ABC) transporters. Mutations in the Bsep gene may lead to progressive familial intrahepatic cholestasis type 2 (PFIC 2) in childhood, a disorder with markedly elevated serum bile salt levels and low content of bile salts in bile, which carries a dismal prognosis. The conjugate export pump (multidrug resistance-associated protein 2, Mrp2) is another ABC-type transporter that has been shown to be capable of transporting divalent sulfated and glucuronidated bile salts.

In experimental animal models of cholestasis induced by application of endotoxin (model for inflammatory cholestasis), ethinyloestradiol (cholestasis of pregnancy), alpha-naphtylisocyanate (vanishing bile duct syndrome), and common bile duct ligation (extrahepatic biliary obstruction), most of the hepatic transporters are down-regulated. Recent findings of up-regulation of basolateral bile salt transporters in cholestasis such as Mrp3 and Mrp4 may provide an escape route for bile salts out of the hepatocyte when apical transport is defective. Cholestasis is also associated with retrieval of canalicular transporters to pericanalicular vesicles. Thus, the function of most hepatobiliary transport systems is presumed to decrease after exposure to cholestatic injury.

Active transport of bile salts across the canalicular membrane represents the rate-limiting step in overall transport from blood to bile and is under both short-term and long-term regulation by posttranscriptional and transcriptional mechanisms, respectively. On the

transcriptional level, hepatic transporters have been shown to be regulated by nuclear receptors (Figure 2). FXR up-regulates Bsep expression in the canalicular membrane of the hepatocyte. An FXR-dependent transactivation has also been demonstrated for the Mrp2 and Oatp8 gene promoter. PXR stimulates expression of Mrp2 and Oatp2. Short-term regulation on the post-transcriptional level occurs via signaling cascades which may involve cytosolic free calcium, adenosine 3,5-cyclic monophosphate (cAMP), phosphatidylinositol 3-kinases, PKC isoforms, and different MAP kinases.

## Bile Salts in Therapy

UDC is a hydrophilic bile salt that is increasingly used for the treatment of various cholestatic disorders. It is normally present in human bile at a low concentration of about 3% of total bile salts. It is the major bile salt in black bear's bile, which has been used in Chinese traditional medicine for the treatment of liver diseases. First reports on the beneficial effects of UDC in patients with liver diseases came from Japan in the 1970s. A number of controlled trials on the use of UDC in primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) have been published in the Western literature since the early 1990s. UDC improves serum liver tests and may delay disease progression to cirrhosis and prolong transplant-free survival in PBC. In PSC, UDC improves serum liver tests and surrogate prognostic markers, but effects on disease progression must further be evaluated. Anticholestatic effects of UDC have also been reported in a number of other cholestatic conditions. After oral administration of unconjugated UDC, 30–60% of the dose is absorbed by nonionic diffusion mainly in the small intestine and to a small extent in the colon and undergoes efficient hepatic uptake and conjugation with glycine and to a lesser extent with taurine. A daily dose of 13–15 mg $kg^{-1}$ UDC causes an enrichment of ~40–50% in biliary bile salts of patients with PBC. The mechanisms underlying the beneficial effects of UDC in cholestatic disorders are increasingly being unraveled. Experimental evidence suggests three major mechanisms of actions: (1) protection of cholangiocytes against cytotoxicity of hydrophobic bile salts, resulting from modulation of the composition of mixed phospholipid-rich micelles, reduction of bile salt cytotoxicity of bile, and possibly, decrease of the concentration of hydrophobic bile salts in the cholangiocytes; (2) stimulation of hepatobiliary secretion, putatively via $Ca^{2+}$- and PKC-dependent mechanisms and/or activation of p38MAPK and extracellular signal-regulated kinases (Erk) resulting in vesicular insertion of transporter molecules (e.g., bile salt export pump, Bsep, and conjugate export pump, Mrp2) into the canalicular membrane of the hepatocyte and, possibly, activation of the inserted carriers; (3) protection of hepatocytes against bile-salt-induced apoptosis, involving inhibition of the mitochondrial membrane permeability transition (MMPT), and possibly, stimulation of a survival pathway.

## See Also the Following Articles

Biliary Cirrhosis, Primary • Cytochrome P-450 • Mitogen-Activated Protein Kinase Family • Protein Kinase C Family • P-Type Pumps: $Na^+/K^+$ Pump

## Glossary

**bile salts** Family of organic anions with a steroid nucleus formed by enzymatic conversion of cholesterol (major human bile salts: cholate, chenodeoxycholate, deoxycholate, lithocholate, ursodeoxycholate).

**bile salt transporters** Membrane carrier proteins mediating the transport of bile salts across cell membranes, among which the $Na^+$-taurocholate-cotransporting polypeptide (Ntcp) at the basolateral hepatocyte membrane, the Bsep at the apical hepatocyte membrane, and the Asbt at the apical membrane of ileocytes represent key carriers for maintenance of an effective enterohepatic circulation of bile salts.

**nuclear hormone receptors** Ligand-activated transcription factors regulating gene expression by interaction with specific DNA sequences. Bile salts have so far been identified to act as physiological ligands of the farnesoid X receptor (FXR), the pregnane X receptor (PXR), and the vitamin D receptor (VDR), thereby modulating their own metabolism and transport.

## Further Reading

Bouscarel, B., Kroll, S. D., and Fromm, H. (1999). Signal transduction and hepatocellular bile acid transport: Cross talk between bile acids and second messengers. *Gastroenterology* **117**, 433–452.

Chiang, J. Y. L. (2002). Bile acid regulation of gene expression: Roles of nuclear hormone receptors. *Endocrine Rev.* **23**, 443–463.

Fuchs, M., and Stange, E. F. (1999). Metabolism of bile acids. In *Oxford Textbook of Clinical Hepatology* (J. Bircher, J. P. Benhamou, N. McIntyre, M. Rizzetto and J. Rodés, eds.) 2nd edition, pp. 223–256. Oxford University Press, New York.

Meier, P. J., and Stieger, B. (2002). Bile salt transporters. *Annu. Rev. Physiol.* **64**, 653–661.

Paumgartner, G., and Beuers, U. (2002). Ursodeoxycholic acid in cholestatic liver disease: Mechanisms of action and therapeutic use revisited. *Hepatology* **36**, 525–531.

Paumgartner, G., Keppler, D., Leuschner, U., and Stiehl, A. (eds.) (2003). *Bile Acids: From Genomics to Disease and Therapy.* Kluwer, Dordrecht, Boston, London.

Russell, D. W. (2003). The enzymes, regulation and genetics of bile acid synthesis. *Annu. Rev. Biochem.* **72**, 137–174.

Trauner, M., and Boyer, J. L. (2003). Bile salt transporters: Molecular characterization, function and regulation. *Physiol. Rev.* **83**, 633–671.

## Biography

Ulrich Beuers is a Professor in the Department of Internal Medicine II, University of Munich, Germany. His principal research

interest is in the field of bile acids and cholestasis with a focus on bile acid-induced modulation of signaling and secretion in liver cells and treatment of chronic cholestatic liver diseases. He holds an M.D. from the University of Freiburg and received his postdoctoral training at the Universities of Göttingen (Department of Biochemistry), Munich (Department of Internal Medicine II), and the Yale University, New Haven (Section of Digestive Diseases).

Thomas Pusl is a resident in the Department of Internal Medicine II, University of Munich, Germany. His special research interests include mechanisms and effects of calcium signals in liver, and mechanisms underlying cholestasis and treatment of chronic cholestatic liver diseases. He holds an M.D. from the University of Munich and received his postdoctoral training at the University of Munich (Department of Internal Medicine II), and the Yale University, New Haven (Section of Digestive Diseases).

# Biliary Cirrhosis, Primary

**Marshall M. Kaplan**
*Tufts-New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts, USA*

**Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease that is characterized by a continuing destruction of interlobular bile ducts, progressive fibrosis, and the eventual development of cirrhosis and liver failure. In contrast to patients seen more than a decade ago, most of whom had symptoms of fatigue, pruritus (itching), or jaundice, more than 60% of PBC patients who are diagnosed now are asymptomatic. Asymptomatic patients live longer than symptomatic ones. However, most asymptomatic patients eventually develop symptoms and their survival is reduced compared to an age- and sex-matched healthy population. There is general agreement that all patients with PBC should be treated but no agreement about the best treatment or even whether any treatment prolongs survival free of liver transplantation. Most physicians initiate treatment with ursodeoxycholic acid (UDCA), the only treatment for PBC approved by the U.S. Food and Drug Administrations (FDA). Some, including the author, believe that the addition of colchicine and/or methotrexate to those who fail to respond fully to UDCA provides an added benefit.**

## Epidemiology

Primary biliary cirrhosis (PBC) was once considered a rare disease. However, increased awareness of PBC has led to earlier and more frequent diagnoses. In some series, it accounted for almost 2% of deaths due to cirrhosis. In a study from England, there was an annual incidence of 5.8 cases per million population and a point prevalence of 54 cases per million. The prevalence in the United States is at least that high. Estimates place the number between 50,000 to 100,000 patients. The etiology is unknown, but may be related to altered immunoregulation. The lack of concordance of PBC in identical twins suggests that some triggering event is required to initiate PBC in a genetically susceptible individual. PBC is much more likely to occur in families of patients with an index case compared to the general population. A number of environmental causes have been implicated in the pathogenesis of PBC, including bacteria and viruses. Some of the most compelling evidence for an environmental factor has been derived from an epidemiological study that demonstrated significant geographic clustering of PBC cases in urban areas in northeast England.

## Etiology

### Autoantibodies and Antimitochondrial Antibody

Ninety to 95% of patients with PBC are women, a sex distribution similar to that for other autoimmune diseases such as systemic lupus. There is an increased incidence of autoantibodies including antimitochondrial antibody, antinuclear antibody, and antimicrosomal antibody. Antimitochondrial antibody (AMA) is detected in 95% of patients with PBC. The major autoantibody found in PBC patients has been called anti-M2. It is directed principally against the dihydrolipoamide acyltransferase component (E2) of the branched keto-acid dehydrogenase and pyruvate dehydrogenase complexes on the inner mitochondrial membrane. Other antimitochondrial antibodies that have been described in PBC, anti-M4, anti-M8, and anti-M9, are most likely artifacts and were not found in a study that employed highly purified human mitochondrial proteins as antigens. The relationship between antimitochondrial antibodies and immunologic bile duct injury is not clear. The mitochondrial antigens are not tissuespecific, and their intracellular location should reduce their immunogenicity. Repeated studies have shown that there is no correlation between the presence or titer of AMA and the severity or course of PBC.

### Mitochondrial Antigens and the Cellular Immune System

Although there is little evidence that AMA plays a direct role in the pathogenesis of PBC, an interaction between the mitochondrial antigens and the cellular immune system appears to be important. The E2 antigens stimulate interleukin-2 production by peripheral blood mononuclear cells and by T cells cloned from liver biopsies of PBC patients. E2 antigens are recognized by both helper and cytotoxic T cells in PBC and cytotoxic

 © 2004, Elsevier Inc. All Rights Reserved. 

T cells appear to mediate bile duct epithelial cell necrosis. The mitochondrial antigens are aberrantly expressed on the luminal surface of biliary epithelial cells from PBC patients, but not in control subjects or patients with primary sclerosing cholangitis. These antigens are expressed in bile duct epithelial cells before two other antigens that are also required for T-lymphocyte-mediated cell destruction, human leukocyte antigen (HLA) class II antigens, and the cellular recognition factor, BB1/B7. This chronology suggests that aberrant expression of the E-2 autoantigen on the surface of biliary epithelial cells initiates lymphocytic destruction of bile ducts, the characteristic lesion in PBC.

### Association with Other Autoimmune Diseases

There is an increased association of PBC with other autoimmune diseases, such as thyroiditis, rheumatoid arthritis, calcinosis cutis, Raynaud's phenomenon, esophageal dysmotility, sclerodactylyl and telangiectasia (CREST) syndrome, Raynaud's disease and Sjögren's syndrome. Although circulating immune complexes have been demonstrated in some PBC patients, it is unlikely that they play a role in pathogenesis. Only a small percentage of PBC patients have immune complexes. Some PBC patients have an IgM that can fix complement in the absence of demonstrable antigen binding and will give false positive results with many of the assays used to detect immune complexes.

## Pathophysiology

The signs and symptoms of PBC are due to long-standing cholestasis. There is a gradual destruction of small intrahepatic bile ducts that results in a decreased number of functioning bile ducts within the liver. This, in turn, causes retention within the liver of substances that are normally secreted or excreted into bile, such as bile acids, bilirubin, and copper. The increased concentration of some of these substances (e.g., bile acids) causes further damage to liver cells. Other substances regurgitate from the liver into the blood and soft tissues and cause symptoms such as pruritus. The identity of the substance or substances that cause pruritus is unknown. It is not any of the naturally occurring primary and secondary bile acids. Most data suggest that it is some substance that is secreted into bile and that binds tightly to cholestyramine, a nonabsorbed, quaternary ammonium resin. Possible pruritogens include the naturally occurring opioids. Serum levels of endogenous opioids (endorphins and encephalins) are increased in PBC, and opioid antagonists will relieve itching in some patients. The striking hyperlipidemia and xanthoma formations in some PBC patients are also a consequence of longstanding cholestasis. The impaired secretion of bile in patients with clinically advanced PBC causes a diminished concentration of bile acids within the intestinal lumen. The bile acid concentration may fall below the critical micellar concentration and thus be inadequate for complete digestion and absorption of neutral triglycerides in the diet. This causes the striking fat malabsorption seen in some jaundiced PBC patients, as well as malabsorption of the fat-soluble vitamins A, D, E, and K. Some PBC patients with Sicca syndrome also have pancreatic insufficiency. The pathogenesis of the osteopenic bone disease that occurs in at least 25% of PBC patients is still unclear. Some data suggest that high concentrations of serum bilirubin may impede osteoblast function. The clinically important bone disorder is osteoporosis, not osteomalacia, as was once believed.

## Pathology

PBC is characterized by portal inflammation, the destruction of interlobular bile ducts, progressive fibrosis, and the eventual development of cirrhosis. PBC is divided into four histologic stages, I to IV. The disease is believed to progress from stage I to stage IV. However, the liver is not affected uniformly in PBC. One biopsy specimen may demonstrate lesions ranging from normal to stage IV. By convention, the histologic stage is the highest stage seen in a biopsy. There are two widely used staging systems, those of Scheuer and Ludwig, both well-known liver pathologists. In the Scheuer classification, stage I is defined by the presence of florid, asymmetric destructive bile duct lesions within portal triads (Figure 1). These lesions are irregularly scattered throughout the portal triads and are often seen only on large surgical biopsies of the liver. The damaged bile ducts are often surrounded by dense collections of lymphocytes, histiocytes, plasma cells eosinophils, and occasionally true giant cells, a pathologic lesion that is called a granuloma (Figure 1A). In the Ludwig classification, stage I is defined by the localization of inflammation to the portal triads. In stage II in the Scheuer classification, there are reduced numbers of normal bile ducts within some portal triads and increased numbers of poorly formed bile ducts with irregularly shaped lumens in others, a lesion called atypical bile duct hyperplasia (Figure 2). There is also mononuclear cell inflammation in portal triads and the beginning of portal fibrosis. In stage II in the Ludwig classification, the inflammation and fibrosis extends beyond portal triads and into the surrounding parenchyma. A diminished number of bile ducts in an otherwise unremarkable-appearing needle biopsy of the liver

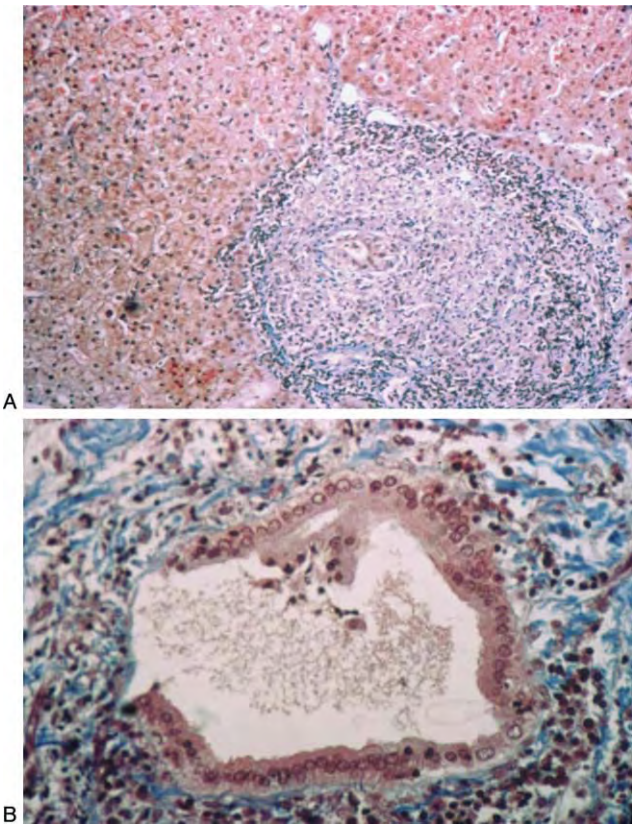



**FIGURE 1** (A) Stage I florid bile duct lesion in primary biliary cirrhosis. A damaged bile duct is at the center of a granuloma (masson trichrome, ×124). (B) Stage I florid bile duct lesion in primary biliary cirrhosis. Epithelial cells have been dislodged from one quadrant of a bile duct (masson trichrome, ×310).

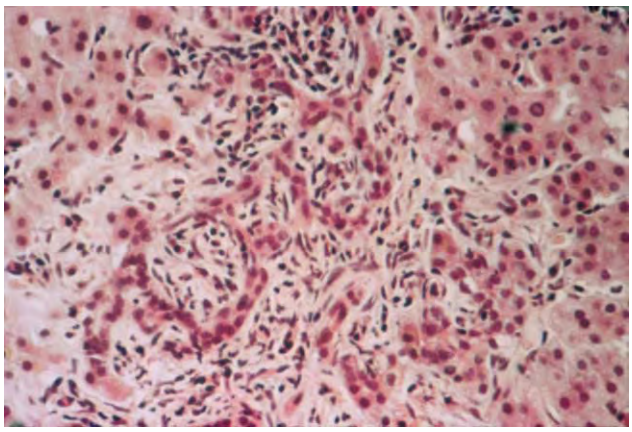


**FIGURE 2** Stage II primary biliary cirrhosis. Atypical bile duct hyperplasia. Bile ducts are tortuous. None is cut in cross section, and there are no visible bile duct lumens (hematoxylin and eosin, ×310).

should alert one to the possibility of PBC. In stage III in both classifications, fibrous septa now extend beyond triads and form portal-to-portal bridges (Figure 3). The portal triads are otherwise similar to those in stage II. Stage IV represents the end stage of the

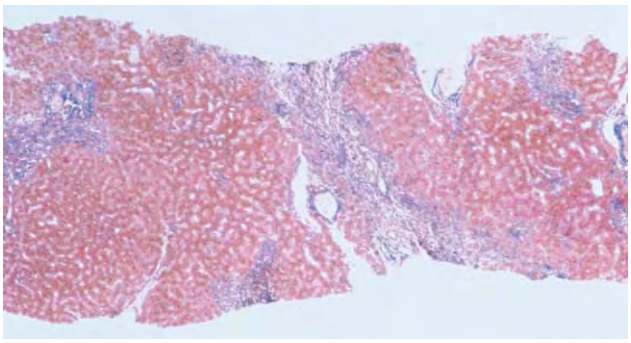


**FIGURE 3** Stage III primary biliary cirrhosis. Adjacent portal triads are linked by septae that contain mononuclear inflammatory cells and scar tissue (masson trichrome, ×310).

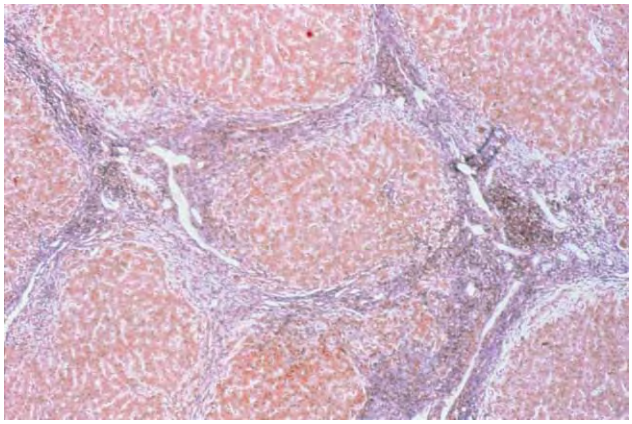


**FIGURE 4** Stage IV primary biliary cirrhosis. Cirrhotic liver removed at the time of liver transplantation performed because of end-stage primary biliary cirrhosis. Nodule formation is evident and there are no visible bile ducts (masson trichrome, ×54).

lesion, frank cirrhosis, and regenerative nodules. It may be difficult to distinguish this late lesion from other types of cirrhosis. A paucity of normal bile ducts in areas of scarring suggests PBC (Figure 4).

## Clinical Findings

The earliest and most common complaint is fatigue. Fatigue, however, is nonspecific and is found in many diseases. The earliest specific complaint in PBC is pruritus, usually worse at bedtime. In some patients, the pruritus begins during the third trimester of pregnancy and persists after delivery. Physical examination may reveal hepatomegaly and increased skin pigmentation. The pigment is melanin, not bilirubin, at this early stage. Excoriations, caused by intense itching and an uncontrollable urge to scratch, may be widespread. Jaundice usually presents later in the course of the disease. Kayser-Fleischer rings have been observed in a few PBC patients with long-standing jaundice.

Unexplained weight loss may also occur. Rarely, patients with PBC may go undetected until late in the course of the disease when they present with severe jaundice, ascites, or bleeding from esophageal varices. During the course of the disease, some jaundiced patients may develop malabsorption and complain of nocturnal diarrhea; frothy, bulky stools; or weight loss in the face of a voracious appetite and increased caloric intake. Fewer than 5% of patients will develop xanthomas (a cutaneous tumor composed of lipid-laden foam cells), but xanthelasmas (a planar xanthoma of the eyelids) are common. Bone pain may occur in PBC patients with osteoporosis, as well as spontaneous collapses of vertebral bodies and hairline fractures of the ribs. Fractures of the long bones are less common.

## Laboratory Tests

Liver function tests reveal a cholestatic pattern. The serum alkaline phosphatase and gamma glutamyl transpeptidase (GGT) are often strikingly elevated. The serum aminotransferases are less elevated and may be normal early in the course of PBC. Serum albumin and prothrombin times are characteristically normal early in the course of the disease. whereas IgM levels are often elevated. The elevated alkaline phosphatase is usually of liver origin. 5′-Nucleotidase and GGT levels parallel the alkaline phosphatase. The serum bilirubin is normal early in the course of the disease, but becomes elevated in most patients as the disease progresses. Both direct and indirect fractions are increased. Despite strikingly high serum cholesterol values, occasionally 800–1600 mg/dl, atherosclerosis is uncommon, most likely because serum high-density lipoprotein (HDL) cholesterol levels are also elevated. Antimitochondrial antibody is positive in 95% of patients. Serum ceruloplasmin is elevated, in contrast to Wilson's disease, another disorder with increased retention of copper within the liver. There is an increased incidence of hypothyroidism in PBC that is not always reflected by routine tests of thyroid function. Elevated thyroid-stimulating hormone (TSH) levels will usually identify such individuals.

## Diagnosis

The combination of a disproportionately elevated alkaline phosphatase and a positive AMA is sufficient evidence to make a diagnosis of PBC as long as the liver biopsy is consistent with this diagnosis. It is prudent to demonstrate that the bile ducts are patent. Ultrasonography is usually adequate for this purpose. Endoscopic cholangiography (ERCP) is recommended if there is a suspicion of bile duct obstruction on imaging tests or if the AMA is negative in a patient with inflammatory bowel disease. The ERCP is done in this setting to detect primary sclerosing cholangitis. A percutaneous needle biopsy of the liver will confirm the diagnosis, allow histologic staging, and provide a baseline for follow-up biopsies that are done to evaluate the efficacy of medical treatment.

## Treatment

### Treatment of Symptoms

#### Pruritus

The nonabsorbed resin, cholestyramine, 4–8 g twice daily with meals, will relieve pruritus in most patients. Commonly prescribed anti-itch medicines such as antihistamines are only helpful early in the course of PBC when itching is not severe. Rifampin, 150 mg twice daily, will control itching in most patients who fail to respond to cholestyramine. Opioid antagonists, such as naloxone and naltrexone, may be effective in the small number of PBC patients who fail to respond to ammonium resins and rifampin.

#### Malabsorption

Some patients with PBC and the Sicca syndrome may have concomitant pancreatic insufficiency. This can be treated with pancreatic enzyme preparations given orally. Malabsorption of fat-soluble vitamins is irregular and unpredictable, but deficiencies of vitamins A, D, E, and K may occur in patients who are chronically jaundiced. Patients with low levels of a specific vitamin should be given supplements of it orally.

#### Anemia and Osteoporosis

PBC patients may develop iron-deficiency anemia. This often reflects unrecognized gastrointestinal (GI) blood loss. Upper endoscopy is indicated to detect esophageal varices or congestive gastropathy. There is no proven effective medical treatment for the osteoporosis other than liver transplantation. Because bisphosphates are an effective treatment for osteoporosis in postmenopausal women and prevent steroid-induced osteoporosis, they are often used in PBC.

### Treatment of the Underlying Disease Process

PBC, if untreated, is a progressive disease that eventually ends in liver failure and the need for liver transplantation. The median survival for symptomatic patients without treatment is approximately 7–10 years. Patients who are asymptomatic at the time of diagnosis have a longer life expectancy than symptomatic patients.

### Medical Treatment

Ursodeoxycholic acid (UDCA), 12–15 mg/kg body weight daily, is the only drug approved by the FDA for the treatment of the PBC. It improves serum bilirubin, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and IgM levels in most patients. It also decreases pruritus and prolongs the time before clinical deterioration and referral for liver transplantation. It is safe and well tolerated. Colchicine 0.6 mg twice daily and/or methotrexate, 0.25 mg/kg body weight per week in three divided doses, have been effective in the author's experience when given to patients who do not respond adequately to UDCA. Combination therapy (UDCA plus colchicine and/or methotrexate) is still under study. At this time, there is still debate about the effectiveness of medical treatment, although the author's experience suggests that medical treatment is effective in most patients, particularly those with precirrhotic PBC. Initiating treatment early in the course of PBC is important because medical therapy is less likely to help once cirrhosis and portal hypertension develop. Liver transplantation is the only effective treatment in patients with clinically advanced disease. The author's current approach is based on the observation that the response to medical treatment in PBC varies among patients. Treatment is initiated with UDCA. Colchicine is added if patients have not responded adequately to UDCA after 1 year. Methotrexate is added if patients have not responded fully to the combination of UDCA and colchicine after another year. An adequate response to treatment consists of the resolution of pruritus, a decrease in serum alkaline phosphatase to values that are $<50\%$ above normal, and improvement in liver histology of at least two points on the necroinflammatory scale. Methotrexate is discontinued after 1 year if patients fail to respond to it. They are maintained on UDCA and colchicine.

### Liver Transplantation

Liver transplantation has improved the survival of PBC patients with cirrhosis and liver failure. Patients who are candidates for liver transplantation are those with cirrhosis and (1) persistent jaundice that is unresponsive to medical treatment, (2) fluid retention, (3) bleeding from esophageal varices, (4) hepatic encephalopathy, or any combination of these four. One-year survival after liver transplantation is greater than 90% in most transplant centers. Survival thereafter resembles a control population matched for age and sex. Recurrence of PBC after liver transplantation is uncommon, but has been seen in a small percentage of patients. It has responded to treatment with ursodiol and colchicine in the author's experience.

## See Also the Following Articles

Bile Salts and their Metabolism • Mitochondrial Auto-Antibodies

## Glossary

**cholestasis** A liver disorder in which bile flow is impeded. Clinical signs of cholestasis are a disproportionate increase in the serum alkaline phosphatase or gamma glutamyl transpeptidase compared to the serum alanine aminotransferase or aspartate aminotransferase. The symptom most characteristic of cholestasis is pruritus.

**hepatic encephalopathy** A disturbance in consciousness that occurs in patients with advanced liver disease. Manifestations can range from subtle changes in personality to frank coma.

**micellar** Pertaining to a colloid particle formed by the aggregation of small molecules. Micelles in bile contain bile acids, phospholipids, and cholesterol.

**mitochondria** Organelles important in energy metabolism that are found in nucleated cells throughout the body.

**necroinflammatory scale** A method of evaluating liver biopsies quantitatively. Scores from 0 to 4 are given for lesions, such as portal inflammation, periportal inflammation, lobular inflammation, bile duct necrosis, and fibrosis, and added together to give a final score.

## Further Reading

Bonis, P. A. L., and Kaplan, M. (1999). Methotrexate in primary biliary cirrhosis unresponsive to ursodeoxycholic acid: An observational study in 10 patients. *Gastroenterology* **117**, 395–399.

Goulis, J., Leandro, G., and Burroughs, A. K., *et al.* (1999). Randomised controlled trials of ursodeoxycholic-acid therapy for primary biliary cirrhosis: A meta-analysis. *Lancet* **354**, 1053–1060.

Hendrickse, M., Rigney, E., Giaffer, M., *et al.* (1999). Low-dose methotrexate is ineffective in primary biliary cirrhosis: Long-term results of a placebo-controlled trial. *Gastroenterology* **117**, 400–407.

Jorgensen, R. A., Dickson, E. R., Hofmann, A. F., Rossi, S. S., and Lindor, K. D. (1995). Characterisation of patients with a complete biochemical response to ursodeoxycholic acid. *Gut* **36**, 935–938.

Kaplan, M. M. (1996). Primary biliary cirrhosis [review]. *N. Engl. J. Med.* **335**, 1570–1578.

Kaplan, M. M., DeLellis, R., and Wolfe, H. (1997). Sustained biochemical and histological remission of primary biliary cirrhosis in response to medical treatment. *Ann. Int. Med.* **126**, 682–688.

Kaplan, M. M., Schmid, C., Provenzale, D., Dickstein, G., and McKusick, A. (1999). A prospective trial of colchicine and methotrexate in the treatment of primary biliary cirrhosis. *Gastroenterology* **117**, 1–9.

Pares, A., Caballeris, L., Rodes, J., *et al.* (2000). Long-term effects of ursodeoxycholic acid in primary biliiary cirrhosis: Results of a double-blind controlled multicentric trial. *J. Hepatol.* **32**, 561–566.

Poupon, R. E., Lindor, K. D., Cauch-Dudek, K., Dickson, E. R., Poupon, R., and Heathcote, E. J. (1997). Combined analysis of randomized controlled trials of ursodeoxycholic acid in primary biliary cirrhosis. *Gastroenterology* **113**, 884–890.

Poupon, R. E., Poupon, R., and Balkau, B. (1994). Ursodiol for the long-term treatment of primary biliary cirrhosis. *N. Engl. J. Med.* **330**, 1342–1347.

Vuoristo, M., Farkkila, M., Karvonen, A. L., *et al.* (1995). A placebo-controlled trial of primary biliary cirrhosis treatment with colchicine and ursodeoxycholic acid. *Gastroenterology* **108**, 1470–1478.

## Biography

Marshall M. Kaplan is the former Chief of the Division of Gastroenterology at Tufts-New England Medical Center and is a Professor of Medicine at Tufts University School of Medicine. His research interests are the etiology and treatment of chronic-cholestatic liver disease. He received his M.D. and M.A.C.P. degrees from Harvard Medical School and the American College of Physicians. He was an associate editor of the *New England Journal of Medicine* from 1993 until 2001, served as Chairman of the Subspecialty Board in Gastroenterology of the American Board of Internal Medicine, and was awarded a mastership by the American College of Physicians.

# Bioenergetics: General Definition of Principles

**David G. Nicholls**
*Buck Institute for Age Research, Novato, California, USA*

**"Bioenergetics" is the study of the molecular mechanisms by which energy, made available by catabolic metabolic pathways or by light capture in photosynthesis, is transformed and made available for cellular processes of growth, motility, and survival. Traditionally, the field has largely focused on studies of bacteria, mitochondria, and chloroplasts, although the principles, largely involving the generation and utilization of ion gradients across membranes, are much more generally applicable.**

## Thermodynamics

Two factors are required for a biochemical reaction to occur spontaneously: the process should involve an increase in the entropy of the system and its surroundings and there should be a mechanism, usually enzymatic, to lower the energy barrier sufficiently to allow the transformation to occur at a significant rate. The entropy difference is the realm of thermodynamics, whereas the energy barrier helps to define the rate of the process.

The simplest thermodynamics deals with the limiting case of isolated systems that exchange neither material nor energy across their boundaries and therefore have no influence on their surroundings. Closed systems exchange energy but not material with their surroundings, whereas real biological systems are open, i.e., exchanging both energy and material (substrates, products). The treatment of open systems is complex and requires non-equilibrium thermodynamics; however, the equilibrium thermodynamics of closed systems can be used to obtain important information, such as the conditions under which a given reaction would be at equilibrium and the extent to which the actual reaction in the cell is displaced from equilibrium. Although equilibrium thermodynamics gives no information as to the rate of a reaction, it can unequivocally exclude any process that is energetically impossible.

### Gibbs Free Energy

The entropic driving force is equally valid for closed and isolated systems; however, analysis is complicated by the effects of the energy transfer across the boundaries of the system affecting the entropy of the surroundings. The Gibbs free energy change, $\Delta G$, is a function that, under conditions of constant temperature and pressure, corrects for the entropy change in the surroundings and enables equilibrium to be calculated just using parameters for the system itself. Gibbs energy changes are used not only to study a reaction in solution, but also to quantify reduction–oxidation (redox) reactions occurring, for example, in the mitochondrial respiratory chain, to calculate the available energy in an ion gradient, and to compare the energy available from absorption of light quanta in photosynthesis.

A basic understanding of bioenergetics is considerably helped by the use of simple analogies. Figure 1 shows schematically the *content* of Gibbs energy ($G$) in a closed system when the reaction A = B is displaced on either side of equilibrium. The slope of the parabola at any point represents the Gibbs energy *change* ($\Delta G$). The bottom of the parabola represents the equilibrium condition: the content of Gibbs free energy is at a minimum and the tangent to the slope, $\Delta G$, is zero. Equilibrium is the lowest energy state and a small conversion of substrate to product at equilibrium causes no free energy change. The left side of the parabola represents reaction conditions that have not yet proceeded as far as equilibrium. The slope $\Delta G$ is negative, which indicates that the reaction can proceed spontaneously (as long as a pathway exists). The further from equilibrium, the greater the slope; i.e., the Gibbs energy change becomes more negative and more energy is available from a conversion of substrate to product at those concentrations. Beyond the equilibrium point, the slope is "uphill." Reactions cannot proceed beyond equilibrium without some independent energy input.

 © 2004, Elsevier Inc. All Rights Reserved. 

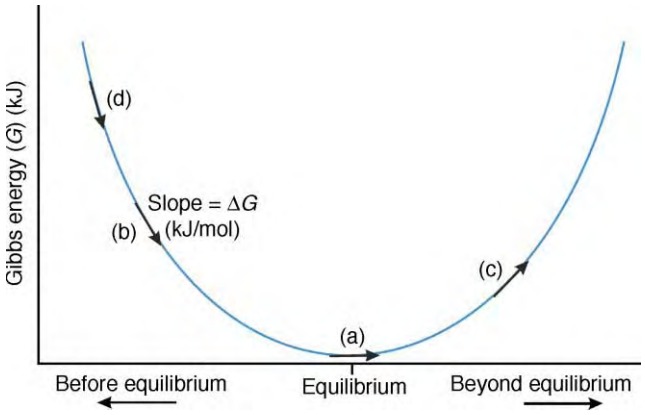



**FIGURE 1** Schematic representation of Gibbs energy in relation to displacement from equilibrium. (a) Gibbs energy ($G$) is at a minimum at equilibrium and Gibbs energy change ($\Delta G$), given by the slope of the parabola, is zero. (b) Before equilibrium, the slope ($\Delta G$) is negative and the reaction can proceed spontaneously. (c) Beyond equilibrium, the slope is "uphill" and an additional energy input would be required. (d) The further the reaction is displaced from equilibrium, the greater the slope; i.e., $\Delta G$ becomes more negative. Adapted from Nicholls, D. G., and Ferguson, S. J. *Bioenergetics* 3 Copyright 2002, with permission from Elsevier.

The Gibbs free energy change can be quantified as a function of the displacement from equilibrium

$$\Delta G(\text{kJ/mol}) = -2.3RT\log_{10}\left(\frac{K}{\Gamma}\right), \qquad (1)$$

where $R$ is the gas constant, $T$ is the absolute temperature, $K$ is the equilibrium constant under the conditions of the determination, and $\Gamma$ is the mass-action ratio of the concentrations of substrates and products measured in the cell or assay mixture. Put more simply, $\Delta G$ changes by approximately 5.7 kJ/mol for each 10-fold displacement from equilibrium. Note that this equation corresponds to the more familiar form that involves standard free energy, e.g.,

$$\Delta G = \Delta G^0 + 2.3RT\log_{10}\left(\frac{[C]^c[D]^d}{[A]^a[B]^b}\right), \qquad (2)$$

but is more logical and symmetrical, emphasizing displacement from equilibrium, rather than the frequently misused abstract concept of standard free energy.

## ATP

ATP synthesis from ADP and phosphate ($P_i$) and the reverse hydrolysis reaction are the dominant reactions in the distribution and utilization, respectively, of Gibbs free energy in the cell. The ATP synthesis reaction is held up to $10^{10}$-fold beyond its equilibrium and thus requires an input of Gibbs free energy of approximately $10 \times 5.7 = 57$ kJ/mol. Conversely, the hydrolysis of 1 mol of ATP can yield up to 57 kJ/mol. There is no magic property associated with ATP (such as the myth of the "high-energy" bond) that fits it for this universal role, except that the equilibrium constant of the reaction is such that a sufficient concentration of ADP remains in a functioning cell to bind to the ATP synthase (see below).

The ATP synthesis reaction is usually expressed in short-hand form, ignoring water, ionization, pH, and chelating ions such as $Mg^{2+}$; i.e.,

$$\text{ADP} + \text{P}_\text{i} = \text{ATP}$$

This may only be used as the basis for thermodynamic calculations if the equilibrium constant is known under *exactly* identical conditions, in which case the common factors cancel out of the ratio $\Gamma/K$ in Eq. (1).

## Redox Potentials

Redox reactions are those that involve coordinate oxidation of one substrate and the reduction of another. An example of a redox reaction occurring in the cytoplasm is that catalyzed by lactate dehydrogenase:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ = \text{Lactate} + \text{NAD}^+. \qquad (3)$$

Redox reactions can be considered hypothetically as the sum of two linked half-reactions:

$$\text{NADH} = \text{NAD}^+ + \text{H}^+ + 2e^- \qquad (4)$$

and

$$\text{Pyruvate} + 2\text{H}^+ + 2e^- = \text{Lactate}. \qquad (5)$$

All biological redox reactions can be considered to involve primary electron transfer, although in many cases the increased negative charge on the reduced component leads to the subsequent binding of one or more protons; thus, ubiquinone reduction to ubiquinol in the respiratory chain involves the addition of two electrons followed by two protons, whereas $NAD^+$ reduction to NADH requires the addition of two electrons followed by one proton [Eq. (5)].

A reduced/oxidized pair, such as NADH/$NAD^+$, is a redox couple and the equilibrium of the half-reaction can be defined in terms of its half-reduction redox potential (the potential at which the concentrations of oxidized and reduced species are equal). A redox couple with a more negative redox potential will tend to oxidize one with a more positive potential. Zero is defined as the potential of a $\frac{1}{2}H_2/H^+$ couple at pH 0 when $H_2$ is 1 atm. Important half-reduction potentials include those for NADPH/$NADP^+$ and NADH/$NAD^+$ (both $-320$ mV), fumarate/succinate ($+30$ mV), ubiquinol/ubiquinone ($+60$ mV), and reduced/oxidized cytochrome $c$ ($+220$ mV). The important $H_2O/\frac{1}{2}O_2$ couple has a redox potential

of + 820 mV when $O_2$ is present at 1 atm and water is 55 *M*.

Redox potentials are not constants, but vary with the state of reduction of the couple. The redox potential of a one-electron reduction couple becomes 60 mV more negative for each 10-fold increase in the reduced/oxidized ratio. Thus, although the *standard* hydrogen electrode at pH 0 has a redox potential of zero, at pH 7 when the proton concentration is only $10^{-7}$ *M*, the redox potential is −420 mV. Two electron reductions change by 30 mV per decade; thus, the NADH/$NAD^+$ couple in mitochondria is typically 10% reduced and thus has a redox potential of approximately −320 + 30 = −290 mV, whereas the NADPH/$NADP^+$ pool is at least 90% reduced and so operates at approximately −350 mV.

Although the historical origin of redox potentials (in electrochemistry) may obscure the relationship, redox changes are governed by Gibbs free energy principles; indeed, the $\Delta G$ associated with the difference in redox potential between two couples can be simply calculated as

$$\Delta G = -nF\Delta E_h, \qquad (6)$$

where $n$ is the number of electrons transferred and $F$ is the Faraday constant (0.965 kJ/mol/mV). As a rough rule of thumb, a "mole" of electrons falling through a potential of 1 V makes 100 kJ of Gibbs free energy available, and 2 mol of electrons passing from NADH to $O_2$ through the respiratory chain corresponds to a $\Delta G$ of −224 kJ. Note that this is compatible with the formation of approximately 3 mol of ATP.

### Ion Electrochemical Potential Differences

The concept of Gibbs free energy as displacement from equilibrium can be extended to gradients of ions and solutes across biological membranes. Two components must be considered: that due to a difference in concentration between the two aqueous compartments and (if the species is charged) that due to the difference in electrical potential between the compartments (usually termed the membrane potential, not to be confused with any surface charge on the membrane itself). The Gibbs free energy is simply the sum of the two components (hence "electrochemical"),

$$\Delta G(\text{kJ mol}^{-1}) = -mF\Delta\psi + 2.3RT\log_{10}\frac{[X^{m+}]_B}{[X^{m+}]_A}, \qquad (7)$$

where $m$ is the charge on the ion, $\Delta\psi$ is the membrane potential in millivolts, and $X^{m+}$ is the concentration of the ion in compartment A or B.

The ion of most interest in bioenergetics is the proton. The equation for the proton electrochemical gradient ($\Delta\mu_{H^+}$) is rather simple, due to the logarithmic nature of pH:

$$\Delta\mu_{H^+}(\text{kJ/mol}) = -F\Delta\psi + 2.3RT\Delta\text{pH}. \qquad (8)$$

The mitochondrial convention is that ΔpH is the pH outside *minus* that in the matrix and is usually negative.

The proton electrochemical gradient is usually expressed in units of voltage. This "proton-motive force," $\Delta p$, is defined as:

$$\Delta p(\text{mV}) = -(\Delta\mu_{H^+})/F = \Delta\psi - 60\Delta\text{pH}. \qquad (9)$$

### Photons

The Gibbs free energy available from a single photon is equal to Planck's constant times frequency, i.e., $h\nu$, where $\nu$ is the frequency of the radiation. A mole (Avagadro's number) of photons corresponds to a $\Delta G$ of 120,000/λ kJ/mol, where λ is the wavelength in nanometers. For 600 nm red light, this corresponds to 200 kJ/mol. Note that the absorption of such a photon would theoretically be capable of lowering (making more negative) the potential of an electron by 2 V.

## The Thermodynamics of Bioenergetic Interconversions

The bioenergetic processes occurring in mitochondria involve the sequential conversion of redox energy into proton-motive force and of proton-motive force into the Gibbs free energy of the ATP pool. Since each is based on Gibbs free energy, the interconversion is very simple. Consider a respiratory chain complex that transfers two electrons through a redox span of $\Delta E_h$ mV. If the complex pumps $n$ protons against a proton-motive force of $\Delta p$ mV, equilibrium would be reached when $n\Delta p = 2\Delta E_h$. For the complex to work in the forward direction, therefore, $n\Delta p = 2\Delta E_h$. Roughly speaking, the forward rate of the complex is proportional to the disequilibrium for small displacements, which is why respiration accelerates when the proton gradient is lowered.

The equilibrium condition for the ATP synthase, where $\Delta p$ is expressed in kilojoules per mole and $\Delta p$ is expressed in millivolts, requires the Faraday constant, $\Delta G_{ATP} = mF\Delta p$, where $m$ represents the number of protons required to generate 1 mol of ATP. Since the ATP synthase is reversible, it can operate in the forward direction (ATP synthesis) when $\Delta G_{ATP} < mF\Delta p$ and in the reverse direction (ATP hydrolysis) when $G_{ATP} > mF\Delta p$.

## Mitochondrial Respiration Rate: The Proton Circuit

A simple but immensely powerful aid to understanding the relationships between proton-motive force, respiration, and proton re-entry pathways is to use a simple electrical analogy (Figure 2). Because there is a fixed stoichiometry between electron flow (and hence respiration) and proton translocation, and because protons must re-enter the mitochondrial matrix at exactly the same rate that they are pumped out, the "proton current," $J_{H^+}$, circulating around the "proton circuit" will equal the rate of respiration times the $H^+/O$ (protons pumped divided by oxygen utilized) stoichiometry of the respiratory chain.

Because the current and potential ($\Delta p$) in the proton circuit are known, Ohm's law can be used to calculate the resistance (or its reciprocal, i.e., the conductance, $C_M H^+$) of the inner membrane to proton re-entry:

$$C_M H^+ = J_{H^+}/\Delta p \qquad (10)$$

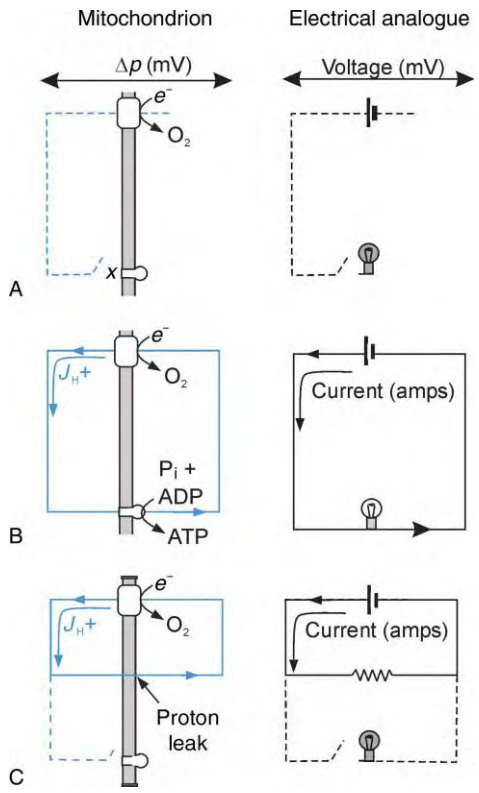



**FIGURE 2** The analogy between the proton circuit and a simple electrical circuit. (A) Open circuit. Zero current (no respiration), potential ($\Delta p$) maximal. (B) Circuits completed, current flows (respiration). Useful work done (ATP synthesized). Potential ($\Delta p$) less than maximal. (C) Short-circuit introduced; energy dissipated, potential low, respiration maximal. Adapted from Nicholls, D. G., and Ferguson, S. J. *Bioenergetics* 3 Copyright 2002, with permission from Elsevier.

Two major components of proton conductance are that which occurs through the ATP synthase, which is tightly coupled to the rate of ATP synthesis, and an endogenous proton leak possessed by all mitochondria. One specialized tissue, brown adipose tissue, possesses an additional controllable proton leak catalyzed by an uncoupling protein that allows respiration, and heat production, to occur without concomitant ATP synthesis.

## See Also the Following Articles

ATP Synthesis in Plant Mitochondria: Substrates, Inhibitors, Uncouplers • Chloroplast Redox Poise and Signaling • Energy Transduction in Anaerobic Prokaryotes • Membrane-Associated Energy Transduction in Bacteria and Archaea

## Glossary

**electrochemical gradient** The driving force inherent in an ion gradient across a membrane, made up of two components: the membrane potential and the ion concentration gradient.

**Gibbs free energy** A thermodynamic term that expresses the extent to which a process is displaced from equilibrium and hence defines its capacity to do work.

**proton circuit** The closed cycle comprising protons pumped across, e.g., the mitochondrial inner membrane and re-entering via the ATP synthase or a leak pathway.

**proton conductance** The conductance of a membrane to protons calculated by applying Ohm's law to the proton circuit.

**redox couple** A reduced/oxidized pair, such as $NADH/NAD^+$.

**redox potential** A thermodynamic measure of the tendency of a redox couple to gain or lose electrons.

## Further Reading

Cramer, W. A., and Soriano, G. M. (2003). Thermodynamics of energy transduction in biological membranes. *In Biophysics Textbook Online*. Available at http://www.biophysics.org/btol/. Biophysical Society.

Nelson, D. L., Cox, M. M., and Lehninger, A. L. (2000). *Principles of Biochemistry*. 3rd edition, Worth, New York.

Nicholls, D. G., and Ferguson, S. J. (2002). *Bioenergetics 3*. Academic Press, London.

## Biography

Dr. David Nicholls is at the Buck Institute for Age Research in Novato, California. His principal research interest is in mitochondrial bioenergetics, with a focus on mitochondrial function/dysfunction in neurons and the relationship to neurodegenerative disease. He holds a Ph.D. from the University of Bristol. He has published articles on brown fat and uncoupling proteins, mitochondrial calcium transport and bioenergetics, and transmitter release of isolated nerve terminals and is co-author of the textbook *Bioenergetics 3*.

# Biotin

**Steven W. Polyak and Anne Chapman-Smith**
*The University of Adelaide, Adelaide, Australia*

**Biotin, or Vitamin H, is a member of the water-soluble class of vitamins. It has been appreciated for some time that biotin is an essential cofactor for a family of enzymes known as the biotin-dependent enzymes. These enzymes are found in all living organisms and catalyze several important metabolic reactions. The cofactor actively participates in the binding and transfer of carbon dioxide between metabolites. Biotin is specifically attached to these enzymes through the activity of another ubiquitous enzyme, biotin protein ligase (BPL). In bacteria, this protein also behaves as a transcriptional repressor of the biotin biosynthetic operon, where biotin itself is an integral component required for DNA binding.**

## Historical Perspective

At the end of the nineteenth century it was observed that extracts from liver and meat could serve as effective treatments for skin lesions induced by a diet of raw egg. Earlier studies had identified a heat stable factor in the same extracts that was also required for yeast growth. In 1935 Kogl and Tannis isolated this factor from egg yolk and called it biotin. The essential growth factor present in the meat extracts, which had become known as Vitamin H (*Haut*, German = skin), was found to be identical to biotin. Later, it was discovered that avidin, a biotin-binding glycoprotein, was the toxic component of raw eggs. The structure of biotin was determined in 1942 and confirmed by chemical synthesis and X-ray analysis.

During the 1950s it became evident that biotin is involved as a cofactor for a class of enzymes that catalyze a variety of carboxyl transfer reactions. It was first demonstrated that $\beta$-methylcrotonoyl-CoA carboxylase, a biotin-requiring enzyme that catalyzes a step in the degradation of leucine, was capable of carboxylating free biotin. It soon became apparent that, in the cell, biotin functions as a cofactor only when bound to protein and that biotin is covalently attached to the $\varepsilon$-amino group of a specific lysine residue of propionyl CoA carboxylase. Subsequently, it was demonstrated for other biotin enzymes that the biotin prosthetic group is attached in the same way.

## Biotin

Biotin, a member of the water-soluble B-complex group of vitamins, is synthesized by higher plants and most fungi and bacteria. Humans, other mammals, and birds cannot synthesize biotin *de novo* and therefore must obtain this essential micronutrient from material synthesized by intestinal microflora and from dietary sources. In mammals, absorption of biotin occurs in the small intestine. Other important sites that play a role in normal biotin physiology include the liver (the principal site of biotin utilization), the kidney (the site of reabsorption of filtered biotin), and the placenta (the site of transport of biotin from the maternal circulation to the developing embryo). Biotin obtained from dietary sources exists in both free and protein-bound forms. Protein-bound biotin is digested by gastrointestinal proteases and peptidases to biocytin and biotin-containing short peptides. Biotin present in these compounds is recycled through the action of biotinidase to release free biotin.

## Biotin Enzymes

Biotin enzymes are a family of enzymes ubiquitously found throughout nature. It appears that all organisms contain acetyl coA carboxylase, reflecting the essential role this enzyme plays in the synthesis of fatty acids required for membrane biogenesis. While some organisms, such as bacteria, possess only one biotin enzyme, other organisms have multiple biotin enzymes. For example, mammalian cells express pyruvate carboxylase, propionyl-CoA carboxylase, and methylcrotonyl-CoA carboxylase in addition to two isoforms of acetyl-CoA carboxylase. These enzymes catalyze key metabolic reactions in gluconeogenesis and amino acid metabolism, in addition to fatty acid metabolism.

The members of this enzyme family utilize biotin as a mobile carboxyl carrier in a conserved reaction

 © 2004, Elsevier Inc. All Rights Reserved. 

mechanism that requires three domains: a biotin carboxylase domain, a transcarboxylase domain, and a biotin domain containing the covalently attached biotin moiety. As shown in Figure 1, these enzymes catalyze reactions in two partial reactions carried out at spatially separate sites on the protein where biotin itself carries a carboxyl group between the two sites. Biotin enzymes can be divided into three classes depending on the nature of the original donor and final carboxyl acceptor. These are the carboxylases (Class I), decarboxylases (Class II), and transcarboxylases (Class III). All eukaryotic enzymes belong to Class I, while prokaryotes contain enzymes from all three classes

The mechanism of biotin carboxylation in the Class I enzymes has been extensively investigated. Experiments on propionyl CoA carboxylase and pyruvate carboxylase showed that the source of $CO_2$ in the reaction is bicarbonate and the reaction is dependent upon the presence of ATP:

$$\text{MgATP} + \text{HCO}_3^- + \text{Enz-biotin} \rightleftharpoons \text{MgADP} + \text{Pi} + \text{Enz-biotin-CO}_2^- \quad (1)$$

At the first partial reaction site, biotin is carboxylated at the 1′-nitrogen probably after the formation of a very labile enzyme-bound carboxyphosphate intermediate that donates the activated carboxyl group to biotin. Although no direct evidence of this reaction mechanism has been reported, several observations suggest this as the most likely scheme. Most notable are the similarities between the biotin enzymes and carbamoyl phosphate synthetase; an enzyme known to utilize a carboxyphosphate intermediate.

Subsequently, in the second partial reaction, the transfer of the carboxyl group from carboxybiotin to the acceptor molecule occurs. It has been proposed that the decarboxylation of carboxybiotin proceeds along a pathway where $CO_2$ and the enolate of biotin are formed. Following protonation, carboxylated biotin can again be formed at the first reaction site under physiological conditions:

$$\text{Enz-biotin-CO}_2 + \text{acceptor metabolite} \rightleftharpoons \text{Enzyme–biotin} + \text{Carboxylated acceptor} \quad (2)$$

The Class II decarboxylases differ from the Class I carboxylases in that the reactions are ATP-independent. The decarboxylases of anaerobic prokaryotes all catalyze decarboxylation of a specific $\beta$-keto acid or acyl-CoA coupled to sodium ion export against a concentration gradient. Thus, this class of enzyme serves as an important energy transducer that does not require ATP. There is only one known member belonging to the Class III biotin enzymes: *Propionibacterium freudenreichii subsp. shermanii* transcarboxylase (TC). TC catalyzes the formation of methylmalonyl-CoA from propionyl-CoA using oxaloacetate as a carboxyl donor, and it exists as a multimeric complex composed of 30 polypeptides of three different types.

## Biotin Domains

The biotin prosthetic group is covalently attached via the $\varepsilon$-amino group of one specific lysine residue within the biotin enzymes. This biotin-accepting lysine is found in a tetrapeptide sequence, Ala–Met–Lys–Met, which is extremely conserved among all biotin enzymes (Figure 2). Additionally, the primary structure surrounding the target lysine residue shows a high degree of homology between a wide range of enzymes and species (Figure 2). Deletion studies have revealed that 30–35 amino acid residues either side of biocytin are necessary to specify biotinylation. Collectively, these structures, able to incorporate biotin *in vivo*, are termed *biotin domains*. The removal of determinants necessary to define the structure of a biotin domain by truncation or mutation results in a molecule that is unable to be biotinylated.

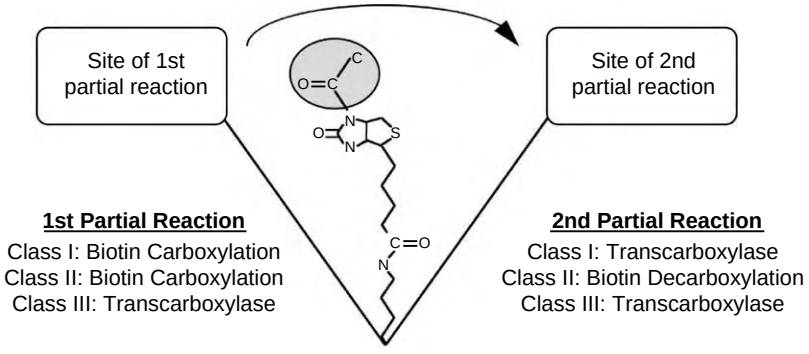



**FIGURE 1** Biotin mediated carboxyl transfer between reaction subsites of biotin enzymes. The biotin group attached to the target lysine is shown located in the cleft of the biotin enzyme. The carboxylate anion, complexed to the biotin, is shown in the shaded circle. The arrow represents movement of the mobile cofactor between the two partial reaction subsites during catalysis. The reactions performed at the separate subsites are indicated for the three classes of biotin enzymes.

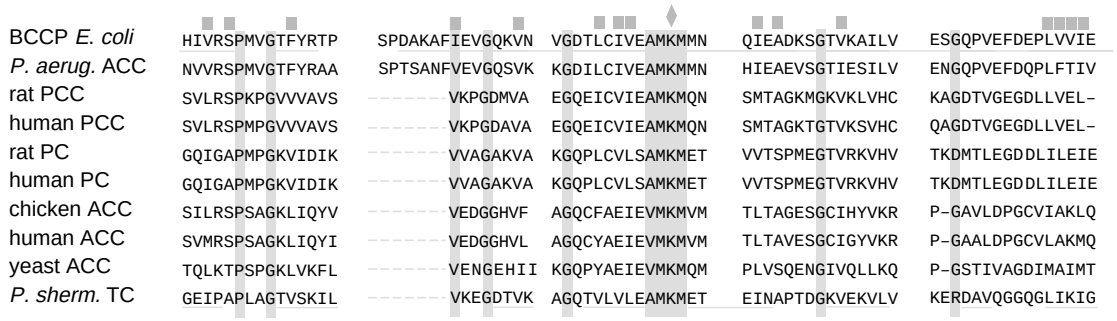



**FIGURE 2** Sequence alignment of the biotin domains of biotin carboxylases from diverse organisms. Residues forming $\beta$-strands in the three-dimensional structures of *Escherichia coli* biotin carboxyl carrier protein (BCCP) and *Propionibacterium freudenreichii subsp. shermanii* transcarboxylase (TC) are underlined, and hydrophobic core residues are indicated by (■). The biotinylated lysine is marked (◆). Shading indicates residues very highly conserved in all biotin domains for which sequence data is available. Reproduced with permission from Chapman-Smith and Cronan (1999), *J. Nutr.* 129, 447S–484S.

The biotin domain participates in a number of heterologous protein:protein interactions in the cell. First, it serves as a substrate in the biotinylation reaction. Here, the biotin prosthetic group is covalently attached to the biotin domain through the enzymatic action of biotin protein ligase (BPL). Subtle conformational changes to the biotin domain that occur upon biotinylation are thought to signal dissociation of the two proteins and yield the biotinylated product. The biotinylated or holo biotin domain is then free to interact with each of the two partial reaction sites in the carboxylase, shuttling carboxyl groups between substrates in the enzyme complex.

The formation of multimeric protein complexes, characteristic of all biotin enzymes, is not necessary for substrate recognition by BPLs. Enzymatic biotinylation experiments, performed using the biotin accepting subunit of the *P. shermanii* TC or the biotin carboxyl carrier protein (BCCP) of *E. coli* acetyl CoA carboxylase, have shown that these domains function equally well as BPL substrates as do the intact or multimeric protein complex. Therefore, the information required for association with BPL is present within the structured biotin domain. BPLs from various sources have been found to recognize and biotinylate acceptor proteins from very different sources.

In 1966, McAllister and Coon first showed that extracts containing BPLs from rabbit liver, yeast, and *P. shermanii* were able to activate enzyme substrates from rabbit and bacteria via the attachment of the biotin prosthetic group. Evidence of cross species recognition *in vivo* was demonstrated when the 1.3S subunit of TC was recombinantly expressed in *E. coli* and shown to be a substrate for the *E. coli* BPL. Truncation analysis of the 1.3S TC subunit revealed that the minimum amount of information required to specify biotinylation was present in the 75 C-terminal amino acid residues. This minimal peptide, fused to $\beta$-galactosidase, was biotinylated *in vivo* by the BPL from *E. coli*. Similarly, the C-terminal 87 amino acid residue of BCCP (BCCP-87) was shown to be a stable protein that can function as a substrate for *E. coli*, insect, yeast, and human BPLs. Together, these studies suggest that all biotin domains fold into an essentially common tertiary structure recognized by all members of the BPL enzyme family.

## Biotin Domain Structure

The structures of two biotin domains have been determined: that of the *E. coli* BCCP-87 (Figure 3) and the 1.3S subunit of *P. shermanii* transcarboxylase. These proteins are structurally related to the lipoyl domains of 2-oxo acid dehydrogenase multienzyme complexes, which also undergo an analogous posttranslational modification. These domains form a flattened $\beta$-barrel structure comprising two 4-stranded $\beta$-sheets with the N- and C-terminal residues close together at one end of the structure. At the other end of the molecule, the biotinyl- or lipoyl-accepting lysine resides on a highly exposed, tight hairpin loop between $\beta$-strands four and five. The structure of the domain is stabilized by a core of hydrophobic residues, which are important structural determinants. Conserved glycine residues (Figure 2) occupy $\beta$-turns linking the $\beta$-strands.

BCCP-87 contains a seven amino acid insertion common to prokaryotic acetyl-CoA carboxylases but not present in other biotin domains. This region of the peptide adopts a "thumb" structure between the $\beta$2 and $\beta$3 strands and, interestingly, forms direct contacts with the biotin moiety in both the crystal and solution structures. The significance of this interaction is not understood, but structural studies on the 1.3S subunit of TC, which is a "thumbless" biotin domain, have revealed that biotin also interacts with the protein. Residues in the N-terminal region of the TC subunit, independent of the biotin domain, functionally compensate for the thumb structure by binding to biotin only when the cofactor is in its carboxylated state.

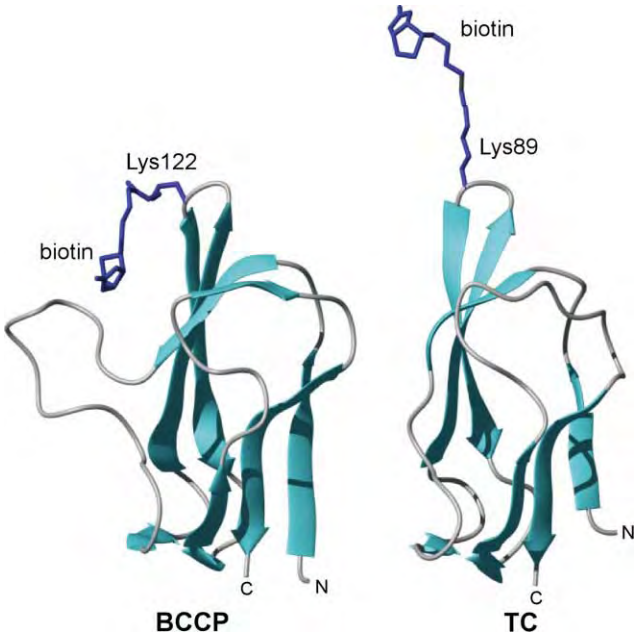



**FIGURE 3** Three-dimensional structures of two biotin domains. The structures of the biotin domains from the biotin carboxyl carrier protein (BCCP) of (left) *Escherichia coli* acetyl CoA carboxylase and (right) the 1.3S subunit of *Propionibacterium freudenreichii subsp. shermanii* transcarboxylase (TC) have been determined. The holo forms of the two proteins with the biotin moiety specifically attached to the target lysine residues at position 122 and 89, respectively, are depicted. Solid arrows represent the $\beta$-strands in the fold. The amino (N) and carboxyl (C) termini of the domain are indicated. The molecules have been orientated to highlight the interaction of biotin with the "thumb" structure in BCCP.

Therefore, it is possible that the mechanism employed by the biotin-enzymes may involve non-covalent interactions between the protein and the prosthetic group.

## Biotin Ligase

### Reaction Mechanism

All BPLs catalyze biotinylation through the same reaction mechanism:

$$\text{Biotin} + \text{MgATP} \rightleftharpoons \text{Biotinyl-5}'\text{-AMP} + \text{PPi} \quad (3)$$

$$\text{Biotinyl-5}'\text{-AMP} + \text{apo-protein} \rightarrow \text{biotinylated protein} + \text{AMP} \quad (4)$$

In the first partial reaction, BPLs bind biotin and ATP in an ordered manner (depending on the species) to form an enzyme:biotinyl-5′-AMP complex or holo-BPL. At this stage, the carboxyl group of inert biotin is activated by the addition of an adenylate group forming the intermediate biotinyl-5′-AMP. In the second partial reaction, holo-BPL complexes with an apo-biotin domain. Nucleophilic attack upon the activated biotin by the amine group of the biotin-accepting lysine residue results in the transfer of biotin from the adenylate onto the biotin domain with AMP acting as the leaving group. This two-step process is analogous to the tRNA synthetase catalyzed linkage of amino acids to the 3′ hydroxyl group of tRNAs.

### *E. coli* Biotin Ligase, BirA

The best characterized BPL is the 35.3 kDa protein BirA from *E. coli*. This protein is a bifunctional molecule which, in addition to biotin protein ligase activity, acts as the transcriptional repressor of the biotin biosynthetic operon. By combining regulation of biotin synthesis and metabolism through the actions of a single protein, bacteria have evolved an exceptional mechanism for maintaining biotin homeostasis. Recent structural studies of BirA, together with enzymatic and genetic studies, have provided powerful insights into BirA action. The crystal structure of BirA shows that the protein is composed of three domains: a DNA binding N-terminal domain, a central catalytic domain, and a C-terminal domain (Figure 4). The central domain contains several poorly defined loops that become more ordered upon binding of biotin or biotinyl-5′-AMP.

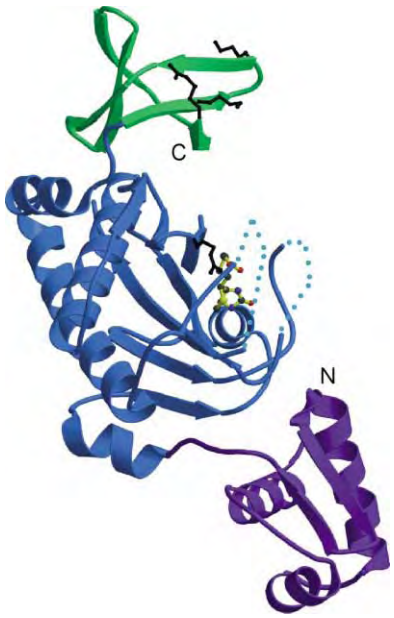



**FIGURE 4** The three-dimensional structure of BirA. The diagram shows the structure of *Escherichia coli* biotin protein ligase, BirA, determined by X-ray crystallography. This figure highlights the three functional domains in the bifunctional protein. The N-terminal domain (N), shown in purple, contains a helix-loop-helix fold to facilitate DNA binding. The central catalytic domain (shown in blue) is depicted with biotin (ball and stick representation) bound at the active site. Dots represent the unstructured loops around the active site that become ordered during the reaction. The function of the C-terminal (C) domain (shown in green) is not fully understood. Modified with permission from Chapman-Smith *et al.* (2001), *Protein Sci.* 10, 2608–2617.

Ordering of these loops in the complex results in the generation of an extended $\beta$-sheet which is required for the interaction with other proteins. Thus, when complexed with biotinyl-5′-AMP, BirA is competent to participate in one of two competing interactions: either with apo-BCCP or with the 40 base pair operator sequence within the biotin synthesis operon. In the presence of apo-BCCP, monomeric BirA preferentially interacts with this protein substrate and catalyzes biotinylation. In the absence of apo-BCCP, the BirA complex forms the homodimer structure required for DNA binding and thus functions as a transcriptional repressor. Therefore, when *E. coli* needs to produce additional active acetyl-CoA carboxylase, its biotin biosynthetic pathway can be derepressed by the expression of apo-BCCP, thereby allowing the bacteria to balance its nutritional requirements.

## See Also the Following Articles

Biotinylation of Proteins • Pyruvate Carboxylation, Transamination and Gluconeogenesis

## Glossary

**biocytin** Biotinylated lysine which is formed between the carboxyl group of biotin and the epsilon amino group on the side chain of lysine.

**biotin** A small, water soluble vitamin belonging to the B-complex group; it is also known as vitamin H.

**biotinidase** A mammalian enzyme that can hydrolyze biotin from either biocytin or short biotin-containing peptides thus facilitating recycling of the vitamin.

**biotin protein ligase** The enzyme [E.C. 6.3.4.15] responsible for covalent attachment of a biotin moiety onto a biotin-enzyme; it is also known as the biotin inducible repressor (BirA) in some prokaryotes and holocarboxylase synthetase in mammalian cells.

## Further Reading

Athappilly, F. K., and Hendrickson, W. A. (1995). Structure of the biotinyl domain of acetyl-coenzyme A carboxylase determined by MAD phasing. *Structure* **3**, 1407–1419.

Attwood, P. V. (1995). The structure and the mechanism of action of pyruvate carboxylase. *Int. J. Biochem. Cell Biol.* **27**(3), 231–249.

Attwood, P. V., and Wallace, J. C. (2002). Chemical and catalytic mechanisms of carboxyl transfer reactions in biotin-dependent enzymes. *Acc. Chem. Res.* **35**(2), 113–120.

Chapman-Smith, A., and Cronan, J. E., Jr. (1999). The enzymatic biotinylation of proteins: A post-translational modification of exceptional specificity. *Trends Biol. Sci.* **24**, 359–363.

McMahon, R. J. (2002). Biotin in metabolism and molecular biology. *Annu. Rev. Nutr.* **22**, 221–239.

Pacheco-Alvarez, D., Solorzano-Vargas, R. S., and Del Rio, A. L. (2002). Biotin in metabolism and its relationship to human disease. *Arch. Med. Res.* **33**(5), 439–447.

Reddy, D. V., Shenoy, B. C., Carey, P. R., and Sonnichsen, F. D. (2000). High resolution solution structure of the 1.3S subunit of transcarboxylase from *Propionibacterium shermanii*. *Biochemistry* **39**, 2509–2516.

Roberts, E. L., Shu, N., Howard, M. J., Broodhurst, R. W., Chapman-Smith, A., Wallace, J. C., Morris, T., Cronan, J. E., Jr. and Perham, R. N. (1999). Solution structures of Apo and Holo biotinyl domains from acetyl coenzyme A carboxylase of *Escherichia coli* determined by triple-resonance nuclear magnetic resonance spectroscopy. *Biochemistry* **38**, 5045–5053.

Weaver, L., Kwon, K., Beckett, D., and Matthews, B. W. (2001). Competing protein: Protein interactions are proposed to control the biological switch of the *E. coli* biotin repressor. *Protein Sci.* **10**, 2618–2622.

Wilson, K. P., Shewchuk, L. M., Brennan, R. G., Otsuka, A. J., and Matthews, B. W. (1992). *Escherichia coli* biotin holoenzyme synthetase/*bio* repressor crystal structure delineates the biotin- and DNA-binding domains. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9257–9261.

## Biography

Steven Polyak completed his graduate studies on biotin protein ligase at The University of Adelaide, Australia, in 1999 before commencing a postdoctoral position at The Hanson Institute. He returned to The University of Adelaide in 2001, where he continues to investigate the protein structure and relationships of BPL.

Anne Chapman-Smith completed a Ph.D. working on pyruvate carboxylase in the early 1980s. Since 1990, she has studied the structure and function of the biotin domains of the biotin carboxylases and their interaction with biotin ligase at the University of Adelaide and the University of Illinois at Urbana – Champaign.

# Biotinylation of Proteins

**Ronald A. Kohanski**
*The Johns Hopkins University, Baltimore, Maryland, USA*

**The biotinylation of proteins is the covalent coupling of biotin to an amino acid or carbohydrate moiety of the protein. Biotinylation occurs in a specific group of proteins known as carboxylases. These are enzymes that are important in several metabolic pathways, including amino acid metabolism, fatty acid biosynthesis, and gluconeogenesis. Each of these carboxylases is biotinylated on a single lysyl residue through the action of a biotin:protein ligase, and are found in all organisms (bacteria, plants, and animals). Biotinylation of proteins is also done by *in vitro* chemical synthesis, whereby biotin can be covalently coupled to any reactive functional group on the protein, and is not necessarily restricted to a single amino acid residue.**

## Analytical and Clinical Applications

Chemically biotinylated proteins are important tools in cell biology, and biochemistry, and have clinical applications. The uses of chemically biotinylated proteins have evolved from the strong interaction between biotin (and its derivatives) and avidin or streptavidin. The medical importance of biotinylated proteins is twofold: (1) life-threatening diseases result from failure to produce adequate levels of endogenous biotinylated proteins. These diseases are particularly serious in newborns and early childhood and (2) the diagnosis and treatment of tumors has been greatly facilitated by chemically biotinylated proteins. As an analytical reagent in cell biology and biochemistry, chemical biotinylation of proteins is in widespread use not only primarily for protein detection and assay, but also for purification. These are considered in greater detail in this article.

## Biosynthesis and Degradation of Natively Biotinylated Proteins

The biotinylated proteins native to cells are the biotin-dependent carboxylases: acetyl CoA carboxylase, beta-methylcrotonyl CoA carboxylase, proprionyl CoA carboxylase, and pyruvate carboxylase. The first is a cytosolic protein and the latter three are in the mitochondria. In bacteria, the biotinylated protein is biotin carboxyl carrier protein which is a subunit of bacterial carboxylases. The biosynthesis of naturally biotinylated proteins occurs in two steps. Biotin is activated by reaction with adenosine triphosphate (ATP), releasing pyrophosphate and biotinyl $5'$-adenosine monophosphate. The hydrolysis of pyrophosphate makes this step essentially irreversible under physiological conditions. The transfer of biotin to an $\varepsilon$-amino group of a specific lysyl residue in the acceptor protein is catalyzed by holocarboxylase synthetase (HS) in eukaryotes, and biotin protein ligase (BPL) in bacteria. The specific lysyl residue that is biotinylated is found within a highly conserved structural motif of the apoprotein – the biotin domain – that ranges in length from 67 to 85 amino acids. The biotin domain is an independently folding motif with a core structure of eight $\beta$-strands forming two apposing antiparallel $\beta$-sheets. Conservation of the catalytic portions of the HS/BPL enzymes and of the biotin domains are so high across evolution that bacterial BPL can recognize and biotinylate mammalian biotin domains, and mammalian HS can recognize and biotinylate bacterial biotin domains. The consensus amino acid sequence surrounding the acceptor lysyl residue is Glu-Ala-Met-Lys-Met, but only the Met-Lys-Met residues are invariant. The conservation of the methionyl residues appears to be dictated by evolutionary pressure to retain a catalytically active carboxylase rather than a recognition site for the synthetase, since Thr or Val can be substituted for these Met residues with little loss of biotinylation but more significant losses in catalytic efficiency of the biotinylated product. In general, it appears that conservation of the $\beta$-sheet structure of the biotin domain is as critical for recognition by the ligase as the consensus sequence, but the latter is governed by the need to conserve optimal enzymatic activity in the biotinylated proteins. Thus the two aspects controlling specificity of protein biotinylation are: first, recognition by the ligase and second, enzymatic activity of the biotinylated protein. Biotinylated proteins are degraded by cellular proteases and the biotinylated lysyl residue (biocytin) is the product of that degradation. Biotinidase is the enzyme that cleaves biocytin to biotin and lysine.

 © 2004, Elsevier Inc. All Rights Reserved. 

## Medical Importance of Biotinylated Proteins

Diseases caused by deficiencies in the biotin-dependent carboxylases fall into two general categories based on whether biotin administration is an effective treatment. Those not treatable with biotin are due to genetic lesions in the biotin-dependent carboxylases (OMIM 200350, 210200, 232050, 232000, and 266150 which are described at the National Institutes of Health website http://www.ncbi.nlm.nih.gov/omim), whereas those treatable with biotin are the result of genetic lesions in the synthetase (multiple carboxylase deficiency; OMIM 253270) or biotinidase (OMIM 25360). Clinically the synthetase and biotinidase deficiencies are distinguished by the early (up to three months post-partum) versus late onset of symptoms, respectively. Mutations in holocarboxylase generally cause a raised Michaelis constant for biotin. Thus the treatment with pharmacological doses of biotin is probably successful because the enzyme only requires higher concentrations of biotin for efficient biotinylation of the apoprotein carboxylases. The success of biotin administration when biotinidase is lost indicates the biological importance of recycling biotin from biocytin to maintain sufficient levels of carboxylase activity. The other clinically significant aspect of biotinidase is its ability to degrade radiolabeled biotin derivatives, which are used in conjunction with biotinylated antibodies to image and/or treat tumors.

## Chemical Synthesis of Biotinylated Proteins

The usual goal of protein biotinylation is to detect the modified protein with avidin, where the avidin is itself linked to a reporter group (a radionuclide, chromophore, fluorophore, or an enzyme). The detection of biotinylated proteins has been important in Western blots in order to determine the expression of proteins in diverse cells exposed to different stimuli or growth conditions, for the immunocytochemical and immunohistochemical localization of proteins within subcellular compartments or in tissues, and for the identification and tracking of surface-biotinylated proteins. In addition, the immobilized avidin (and derivatives) can be used for affinity capture and purification of biotinylated proteins, or in the capture of receptors using biotinylated ligands. Biotinylation of proteins can be achieved through covalent attachment of a biotin derivative to any freely reactive functional group on a protein. The most commonly used sites include primary amines ($\alpha$- or $\varepsilon$-amino groups of the N-terminal or lysyl residues, respectively, as illustrated in Figure 1B), thiol

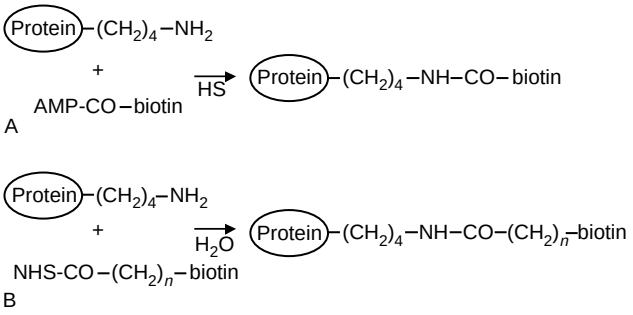



**FIGURE 1** Biotinylation of proteins by enzymatic and chemical reactions. (A) The transfer of biotin from AMP to the $\varepsilon$-amino group of a lysyl residue in a protein, catalyzed by holocarboxylase synthetase (HS) forming an amide linkage. (B) The transfer of biotin, with a hydrocarbon spacer of "n" methylene groups from an N-hydroxysuccinimide ester (NHS) to an $\varepsilon$-amino group of a protein in water.

groups (of cysteinyl residues), and carbohydrate groups. The reactive derivatives of biotin for these reactions use N-hydroxysuccinmide esters, iodoacetyl, or disulfide derivatives of biotin, and hydrazide derivatives of biotin, respectively. The critical factor in the useful biotinylation of proteins is allowing avidin to bind the biotinyl group after conjugation. This requires sufficient distance between the surface of the biotinylated protein and avidin, since biotin is bound in a fairly deep pocket in avidin. This problem was solved by Klaus Hofmann, who found that an $\varepsilon$ aminocaproyl group offered a sufficiently long spacer group for this purpose, as illustrated in Figure 1. The use of sulfo-N-hydroxysuccinimidyl-esters (sulfo-NHSH esters) for biotin derivitization introduced by James Staros further simplified the use of biotin derivatives because it increased the water-solubility and amine-specificity of the reactive ester. Importantly, the sulfo-NHS esters cannot cross cell membranes, and this permits the selective labeling of cell surface proteins through biotinylation of their extracellular domains.

## Clinical Application of Biotinylated Antibodies

Clinically, biotinylated antibodies are in current use for the detection and treatment of tumors, with success in many cases. The multistep procedure can be summarized as follows: biotinylated antibodies against a tumor-specific antigen are introduced into circulation. Binding to the antigen concentrates the biotinylated antibody at the site of the tumor. Avidin or strepavidin is introduced, which binds the pretargeted antibody. Because these are tetramers, at least one biotin-binding site is expected to remain unoccupied in the avidin:biotinylated antibody complex.

A biotin derivative that chelates a radionuclide is then introduced, which is trapped by the open biotin-binding sites on avidin at the site of the tumor. This protocol can be used to visualize the tumor *in vivo* by immunoscintigraphy or positron emission tomography. With the appropriate radionuclide, radiotherapy of the tumor is also achievable. In such protocols, circulating biotinidase plays a counter-productive role, since it can and does break down biocytin analogues. Thus knowledge of the "biotin cycle" of endogenously biotinylated proteins has been important in the clinical application of chemically synthesized biotinylated proteins.

## See Also the Following Articles

Biotin • Pyruvate Carboxylation, Transamination and Gluconeogenesis

## Glossary

**avidin** A protein that binds "avidly" to biotin ($K_d < 10^{-15}$ M) and is found in egg whites. A related bacterial protein is streptavidin, from *Streptomyces avidinii*.

**biocytin** A biotin derivative formed between biotin and lysine. It is formed by the catabolism of carboxylases, and is itself degraded to biotin and lysine by the enzyme biotinidase.

**biotin** A water-soluble vitamin that is used by carboxylases for transfer of $CO_2$ groups in biosynthesis. It is bound tightly by the proteins avidin and streptavidin.

## Further Reading

Boerman, O. C., van Schaijk, F. G., Oyen, W. J. G., and Corstens, F. H. M. (2003). Pretargeted radioimmunotherapy of cancer: Progress step by step. *J. Nucl. Med.* **124**, 400–411.

Luna, E. J. (2001). Biotinylation of proteins in solution and on cell surfaces. In *Current Protocols in Protein Science* (J. E. Coligan, B. M. Dunn, H. L. Ploegh, D. W. Speicher and P. T. Wingfield, eds.) 1st edition, Vol 3, supplement 6, pp. 3.6.1–3.6.15. Wiley, New York.

Wilchek, M., and Bayer, E. A. (eds.) (1990). Avidin-biotin technology. In *Methods in Enzymology*, Vol 184. Academic Press, San Diego, CA.

Wilchek, M., and Bayer, E. A. (eds.) (1999). (Strept) Avidin-biotin system. In *Biomolecular Engineering*, Vol 16.

Wolf, B. (2001). Disorders of biotin metabolism. In *The Metabolic and Molecular Bases of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, eds.) 8th edition, Vol 3, pp. 3935–3962. McGraw-Hill, New York.

## Biography

Ronald Kohanski received his Ph.D. in Biochemistry in 1981 at the University of Chicago. He was a member of the Department of Biochemistry at the Mount Sinai School of Medicine in New York, from 1986 to 2002, and is currently on the faculty at Johns Hopkins University, in the Department of Pediatrics.

# Bradykinin Receptors

**Ronald M. Burch**
*AlgoRx Pharmaceuticals, Inc., Cranbury, New Jersey, USA*

**Bradykinin is an inflammatory nonapeptide, whose generation in tissues and body fluids elicits numerous physiological effects including vasodilation, edema, smooth muscle contraction, as well as pain and hyperalgesia, by stimulating A- and C-neurons. Bradykinin contributes to inflammatory responses in acute and chronic diseases including allergic reactions, arthritis, asthma, sepsis, viral rhinitis, and inflammatory bowel diseases.**

## Overview

Most G protein agonists are hormones or neurotransmitters synthesized intracellularly and stored in secretory granules prior to release. In contrast, bradykinin is a member of the kallikrein–kininogen–kinin system, a complex of two substrates (kininogens) activated by two enzymes (kallikreins) to produce four inflammatory mediators (kinins) that bind to two bradykinin receptors (Figure 1). The substrates, high molecular weight (HMW) kininogen and low molecular weight (LMW) kininogen, are $\alpha_2$ macroglobulins produced by alternative splicing of the same mRNA. HMW kininogen circulates in blood in a 1:1 complex with plasma prekallikrein. Upon tissue injury, factor XII, a component of the coagulation cascade, cleaves plasma prekallikrein to activate it. Plasma kallikrein acts on HMW kininogen to release bradykinin (plasma kallikrein also activates LMW kininogen to produce bradykinin). Tissue kallikrein is synthesized by many types of epithelial cells and secretory cells. Thus, unlike plasma kallikrein, it acts locally. Tissue kallikrein acts only on LMW kininogen and cleaves it one residue to the amino terminus of the site of cleavage of plasma kallikrein to produce a decapeptide, kallidin (or Lys-bradykinin). Bradykinin and kallidin are equipotent agonists of bradykinin B2 receptors and are usually referred to generically as bradykinin or kinins. It should be noted that bradykinin and kallidin are chemically heterogeneous since the proline residues within the peptides may be partially hydroxylated.

Kinins are metabolized further by a variety of enzymes, most of which inactivate them. The major inactivating enzyme is kininase II, especially prevalent in the lungs and in vascular beds, and its activity is largely responsible for the very short half-life of the kinins, about 15 seconds. Kininase II removes the carboxyterminal dipeptide from both bradykinin and kallidin. Kininase II is also known as an angiotensin-converting enzyme because it converts angiotensin I into the vasoconstrictor angiotensin II. Of importance to the present discussion, another enzyme, kininase I, removes the carboxyterminal Arg from bradykinin and kallidin to produce the other two inflammatory mediators, the bradykinin B1 receptor agonists desArg$^9$-bradykinin and desArg$^{10}$-kallidin, respectively. Like the agonists of B2 receptors, bradykinin and kallidin, the desArg metabolites have similar potency as agonists of B1 receptors.

Nearly all tissues express B2 receptors, and bradykinin-induced activation of B2 receptors is implicated in many disease states. B1 receptors are expressed in only a few tissues under normal conditions and only in very small numbers. Several disease states are associated with rapid induction of B1 receptors in specific tissues.

## B1 Receptor

The existence of a B1 receptor activated by what had been considered an inactive bradykinin metabolite, desArg$^9$-bradykinin, was a provocative proposal. This receptor was not found in normal tissues, but was found to be induced during incubation of tissues *in vitro*. Identification of a synthetic B1 receptor antagonist, desArg$^9$, Leu$^8$-bradykinin, added credence to the existence of a physiologic receptor. Later, induction of B1 receptors was found pharmacologically to be associated with specific disease processes *in vivo*, and in 1994, the existence of the B1 receptor was conclusively confirmed with the cloning of the receptor protein.

B1 receptors have been implicated in hyperalgesia, plasma extravasation, white blood cell activation and accumulation, and in the control of blood pressure. However, it seems certain that complete understanding of the roles of this inducible receptor in pathophysiology is not at hand. B1 receptors from several species have been cloned. They are typical G protein-coupled

 © 2004, Elsevier Inc. All Rights Reserved. 

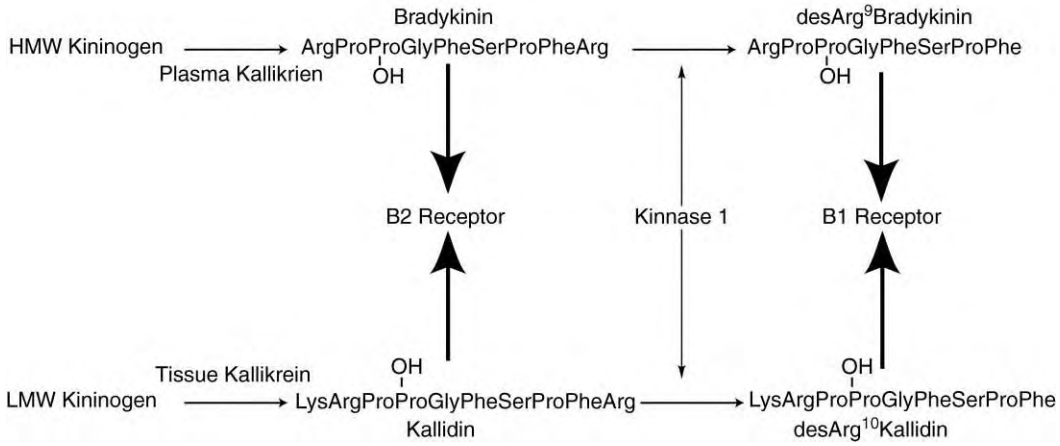



**FIGURE 1** Formation of endogenous bradykinin receptor ligands. Bradykinin and kallidin bind to B2 receptors, whereas, desArg$^9$-bradykinin and desArg$^{10}$-kallidin bind to B1 receptors. Both bradykinin and kallidin may contain some proline residues that are hydroxylated. If hydroxylated, the most common residue is Pro$^3$ in bradykinin and Pro$^4$ in kallidin (shown hydroxylated in the figure).

receptors in sequence. The human receptor has a predicted sequence of 353 amino acids and is only 36% homologous to B2 receptors. The B1 receptor activates phosphatidylinositol-specific phospholipase C and possibly phospholipase $A_2$.

The B1 receptor gene is composed of three exons. The entire coding region for the receptor is contained within the third exon. A variety of polymorphisms have been identified. One, a G/C single nucleotide polymorphism in the promoter region has been associated with disease. Expression of the C allele is higher than the G allele, and patients with the G allele have greater incidence of inflammatory bowel disease and end-stage renal disease.

## B1 Receptor Regulation

Most G protein-coupled receptors are internalized quickly after the binding of their ligands. B1 receptors, however, are not internalized following binding of desArg-kinins. This results in very prolonged activation of signal transduction, which translates into rather few receptors per cell being necessary for biological activity when compared to many other G protein-coupled receptors. Ligand binding studies in a variety of tissues reveal only hundreds to a few thousand B1 receptors per cell, whereas most G protein-coupled receptors, including B2 receptors, are found in the tens of thousands per cell.

In contrast to prolonged phosphatidylinositol turnover, B1 receptor-mediated arachidonic acid release and prostaglandin synthesis is short-lived, which is similar to B2 receptors. The mechanism for this differential desensitization is unknown, but it may be linked to sequestered pools of arachidonate-containing phospholipids or phosphorylation of an intracellular binding site for a transduction protein.

B1 receptors have been shown by immunoprecipitation studies to couple to $G\alpha q$ and $G\alpha i$. These G proteins commonly couple receptors to phosphatylinositol-specific phospholipase C and elevation of intracellular free calcium activity. Activation of B1 receptors leads to elevation of intracellular free calcium activity by increasing calcium entry into the cell; this is in contrast to the B2 receptor which acts primarily to release bound intracellular calcium.

## Regulation of B1 Receptor Induction

Unlike many G protein-coupled receptors, the primary regulation of the B1 receptor number appears to occur by transcriptional induction. Biological studies have found that B1 receptor expression is induced by a variety of cytokines including endotoxin, interleukin 1$\beta$, and tumor necrosis factor. The specific domains affected by these stimulating ligands have not been identified, but activation of B1 receptor transcription has been correlated to activation of the transcription factor NF-$\kappa$B.

The regulation of the B1 receptor gene has also been shown to be regulated by stabilization of mRNA. Interleukin 1 treatment of cells has been demonstrated to double the half-life of B1 receptor mRNA. In addition, protein synthesis inhibitors increase B1 receptor gene stability, suggesting the existence of a short-lived protein that causes destabilization. This protein may interact with the 3′ untranslated region, since experimentally altering the 3′ untranslated region results in B1 receptor mRNA's with varying half-lives.

### B1 Receptor Knockout Animals

B1 receptor knockout mice have been reported to develop normally and to have normal blood pressure. In contrast, when inflammatory stimuli are applied, a dramatic reduction in accumulation and apoptosis of neutrophils have been reported, as well as hypoalgesia; this is consistent with pharmacological evidence presented in the past.

## B2 Receptors

B2 receptors mediate bronchoconstriction, local blood flow regulation, hypotension, acute inflammatory reactions, pain, and hyperalgesia. B2 receptors from several species have been cloned. Like B1 receptors, they are typical G protein-coupled receptors in sequence. The human receptor has a predicted sequence of 364 amino acids. Both B1 and B2 receptors are found on chromosome 14 in humans, only about 12 kb apart. Like B1 receptors, B2 receptors activate phosphotidylinositol-specific phospholipase C. In addition, in most tissues, B2 receptor activation results in the production of prostaglandins and other arachidonic acid metabolites.

The B2 receptor gene is composed of three exons, of which exon 2 and exon 3 provide the coding region for the receptor. Bradykinin has been implicated in hypertension for decades. To date, nearly 80 single nucleotide polymorphisms have been identified for the B2 receptor. The promoter region for the B2 receptor contains a single nucleotide polymorphism, T/C. The C allele has been demonstrated to be an independent risk factor for essential hypertension in several ethnic groups. Several other alleles have been reported and were found to be differentially associated with several disease states. Expansion of these observations promises the potential to define the role of bradykinin in a variety of physiologic and pathophysiologic states.

### B2 Receptor Regulation

In contrast to the B1 receptor, B2 receptor number does not seem to be significantly regulated by inducible gene expression. Instead, B2 receptors are constitutively expressed, as is the case for many G protein-coupled receptors. Activation of the B2 receptor by binding of bradykinin results in rapid internalization of the receptor protein by endocytosis, occurring within a few minutes. Internalization results in cessation of the biological activity of the receptor. Thus, activation of a particular B2 receptor results in a transient increase in intracellular calcium activity and transient prostaglandin release. Receptors are recycled intracellularly, with stripping of bradykinin and the return of a reactivated B2 receptor to the cell surface where it may be reactivated. Most G protein-coupled receptors are internalized following attachment to arrestin and dynamin-dependent clathrin-coated vesicles. In contrast, the B2 receptor becomes associated with caveolae.

### B2 Signal Transduction

B2 receptors have been demonstrated to activate all of the signal transduction mechanisms described for G protein-coupled receptors (Figure 2). Any given cell type supports only a few of the transduction pathways, and these are primarily controlled by the specific G proteins expressed by the given cell type. B2 receptors have been shown by immunoprecipitation studies to couple to G$\alpha$q, which mediates interaction with phosphatidylinositol-specific phospholipase C. B2 receptors have also been shown by immunoprecipitation to couple to G$\alpha$i. G protein depletion studies have suggested that G$\alpha$i(2) and G$\alpha$i(3) couple B2 receptors to arachidonic acid release, presumably through the activation of phospholipase $A_2$. In contrast to B1 receptors, activation of B2 receptors results in increased intracellular calcium activity from the release of calcium from intracellular stores.

### B2 Receptor Knockout Animals

Studies with B2 receptor knockout mice have shown that the animals develop normally. However, one study found that when animals are fed a high-salt diet, severe hypertension occurs. It has also been reported that the

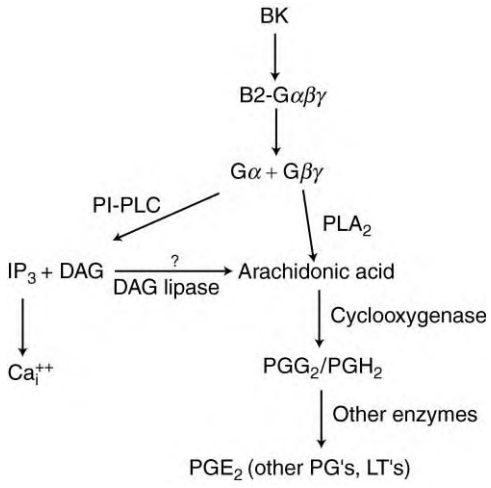



**FIGURE 2** Signal transduction elicited by B2 receptors: BK, bradykinin or kallidin; $PLA_2$, phospholipase $A_2$; $IP_3$, inositol trisphosphate; DAG, diacylglycerol; PG, prostaglandin; LT, leukotriene. Not shown is phospholipase D, which in some cells provides a shuttle for arachidonic acid to appropriate subtrates for hormone-sensitive phosphlipase $A_2$. In most cells, bradykinin does not directly alter cAMP concentration; often, this occurs as a consequence of $PGE_2$ binding to its receptor.

renin–angiotensin system was abnormal (the kallikrein–kinin system and the renin–angiotensin system are antagonistic hemodynamic regulatory systems that play important roles in blood pressure homeostasis) and abnormal renal development occurred. A variety of cardiac defects have also been reported as well as chronically elevated heart rate.

## Other Bradykinin Receptors

Many pharmacologic studies using bradykinin analogs have yielded data suggestive of additional bradykinin receptors, or at least heterogeneity of bradykinin receptors. For example, in smooth muscle preparations there is evidence for expression of different B2-like receptors expressed by the smooth muscle and the neuronal endings in the smooth muscle. Similarly, "B3" receptors have been reported in trachea, and "B4" and "B5" receptors have been reported in opossum esophagus. A criticism of all of these studies is the use of rather weak bradykinin analogs, with dissociation constants of several nM, compared to pM dissociation constant for bradykinin itself. No genetic evidence for additional receptors beyond B1 and B2 has been obtained to date.

## Bradykinin Receptor Antagonists in Clinical Medicine

While highly potent peptide antagonists for B1 receptors have been described, none to date have been evaluated in clinical trials. In contrast, several peptide antagonists for B2 receptors have been evaluated as anti-inflammatory agents, including NPC 567 and Hoe 140. In addition, several nonpeptide antagonists of B2 receptors have been described. An issue that has slowed clinical development of such antagonists is the observation that bradykinin appears to play an important role in coronary artery function. It has been suggested that antagonism of B2 receptors in patients with coronary artery disease may have deleterious effects during ischemic events, perhaps even triggering myocardial infarction.

### See Also the Following Articles

Angiotensin Receptors • G Protein-Coupled Receptor Kinases and Arrestins • Inositol Phosphate Kinases and Phosphatases • Phospholipase $A_2$ • Prostaglandins and Leukotrienes

### Glossary

**angiotensin** Peptide produced by the action of the enzyme renin on the protein angiotensinogen.

**G protein** Trimeric complex of $\alpha$, $\beta$, and $\gamma$ subunits. Activation of a G protein-coupled receptor results in dissociation of the G$\alpha$ subunit, which in turn activates signaling proteins such as adenylyl cyclase, certain ion channels, and phospholipases. The activity of the G$\alpha$ subunit is terminated by endogenous GTPase activity, followed by reassociation of the G$\alpha$ with G$\beta\gamma$ subunit.

**ischemia** Loss of blood flow with resultant metabolic insufficiency of a tissue bed.

**myocardial infarction** Death of heart muscle following prolonged, complete ischemia.

**prostaglandin** Autocoid (local hormone) produced by the action of cyclooxygenase 1 or cyclooxygenase 2 on arachidonic acid. A variety of prostaglandins may be produced, each with specific biological actions elicited by binding to cell-surface receptors.

**single nucleotide polymorphism** Difference at a specific place in a DNA sequence of a single nucleotide. The two gene variants are alleles.

### Further Reading

Ausitn, C. E., Faussner, A., Robinson, H. E., Chakravarty, S., Kyle, D. J., Bathon, J. M., and Proud, D. (1997). Stable expression of the human kinin B1 receptor in chinese hamster ovary cells. *J. Biol. Chem.* **272**, 11420–11425.

Burch, R. M. (ed.) (1991). *Bradykinin Antagonists. Basic and Clinical Research*. Dekker, New York.

Burch, R. M., and Kyle, D. J. (1992). Recent developments in the understanding of bradykinin receptors. *Life Sci* **50**, 829–838.

Marceau, F., and Bachvarov, D. R. (1998). Kinin receptors. *Clin. Rev. Allergy Immunol* **16**, 385–401.

Prado, G. N., Taylor, L., Zhou, X., Ricupero, D., Mierke, D. F., and Polgar, P. (2002). Mechanisms regulating the expression, self-maintenance, and signaling-function of the bradykinin B2 and B1 receptors. *J. Cell. Physiol* **193**, 275–286.

Regoli, D., and Barabe, J. (1980). *Pharmacol. Rev.* **32**, 1–46.

### Biography

Ronald M. Burch is President and Chief Executive Officer of AlgoRx Pharmaceuticals, Inc. His primary research interests are in the mechanisms of pain transmission and immunotherapeutics, both are areas in which bradykinin play roles. He holds a Ph.D. in pharmacology and M.D., both obtained from the Medical University of South Carolina.

# Branched-Chain α-Ketoacids

**David T. Chuang**
*University of Texas Southwestern Medical Center, Dallas, Texas, USA*

**The branched-chain α-ketoacids (BCKAs) comprise α-keto-isocaproate (KIC), α-keto-β-methylisovalerate (KMV), and α-ketoisovalerate (KIV) that are derived from branched-chain amino acids (BCAAs) leucine, isoleucine, and valine, respectively, through reversible transamination. The oxidative decarboxylation of these three BCKAs is catalyzed by a single branched-chain α-ketoacid dehydrogenase (BCKD) complex to give rise to various branched-chain acyl-CoAs (reaction [1]).**

$$\text{R–CO–COOH} + \text{CoA-SH} + \text{NAD}^+ \rightarrow \text{R–CO–S–CoA} + \text{CO}_2\uparrow + \text{NADH} + \text{H}^+ \quad [1]$$

**In patients with the inherited Maple Syrup Urine Disease (MSUD), the activity of the BCKD complex is deficient. This metabolic block results in the accumulation of BCAAs and BCKAs. Clinical manifestations include neonatal or later onset of often-fatal ketoacidosis, encephalopathy and other acute and chronic neurological dysfunction as well as mental retardation in survivors. A distinct phenotype is the strong maple syrup odor in the urine of patients, and hence the name of the disease. There are currently five clinical phenotypes associated with MSUD: classic, intermediate, intermittent, thiamin-responsive and E3-deficient. The prevalence of MSUD is one in 185 000 live births worldwide.**

## Degradative Pathways of BCAAs

The oxidation of BCAAs leucine, isoleucine, and valine begins with the transport of these amino acids into cells through the $Na^+$-independent L transporter in the plasma membrane. In the cell, the BCAAs undergo the first transamination step catalyzed by BCAA aminotransferases (BCATs), which are either cytosolic or mitochondrial, to produce BCKAs (Figure 1). The second step, i.e., the oxidative decarboxylation of BCKAs catalyzed by the mammalian BCKD complex, occurs exclusively in mitochondria. The reaction products of KIC, KMV, and KIV are isovaleryl-CoA, α-methylbutyryl-CoA, and isobutyryl-CoA, respectively. These branched-chain acyl-CoAs subsequently undergo the third step, i.e., dehydrogenation by specific acyl-CoA dehydrogenases. The dehydrogenation of isovaleryl-CoA is catalyzed by isovaleryl-CoA dehydrogenase; and of α-methylbutyryl-CoA as well as isobutyryl-CoA is carried out by α-methyl branched-chain acyl-CoA dehydrogenase. After these three steps, the degradative pathways for each of BCKAs diverge. Leucine yields acetyl-CoA and acetoacetate as end products, and is therefore a ketogenic amino acid. Valine produces succinyl-CoA, and is accordingly glucogenic. Succinyl-CoA enters the Krebs cycle, and is eventually converted to glucose by gluconeogenesis. Isoleucine is both ketogenic and glucogenic since it is metabolized to acetyl-CoA and succinyl-CoA.

## Interorgan Relationships

Oxidation of BCAAs involves extensive interplay of metabolites between muscle and liver in the rat. This is due to the nonuniform distribution of BCATs and the BCKD complex among organs and tissues. In rat skeletal muscle, BCAT activity is high, but the BCKD complex activity is low. A reverse situation exists in rat liver with respect to levels of the two enzyme activities. Both rat liver and heart degrade BCKAs at high rates, but in hepatectomized rats leucine oxidation is decreased. This led to the prevailing view, based on the rat model, that the primary role of muscle is transamination of BCAAs, which provides the major source of circulating BCKAs. BCKAs are transported to liver, kidney, and heart, where they are oxidized. However, the interorgan shuttling of BCAA metabolites may not occur in humans. It was shown recently that the BCKD complex activity in human liver is similar to that in skeletal muscle. The results confirm an earlier observation that BCKD complex activity in human liver and kidney was markedly lower than in the rat counterparts, but activity in skeletal muscle was similar between two species. The human liver exhibits twice as high BCAT activity as human skeletal muscle. This finding suggests that human liver is capable of oxidizing BCAAs *in situ* and does not depend on BCAT activity in skeletal muscle for the conversion of BCAAs to BCKAs. Since skeletal muscle comprises 40% of the body mass, this tissue is likely the major site for BCAA oxidation in humans.

 © 2004, Elsevier Inc. All Rights Reserved. 

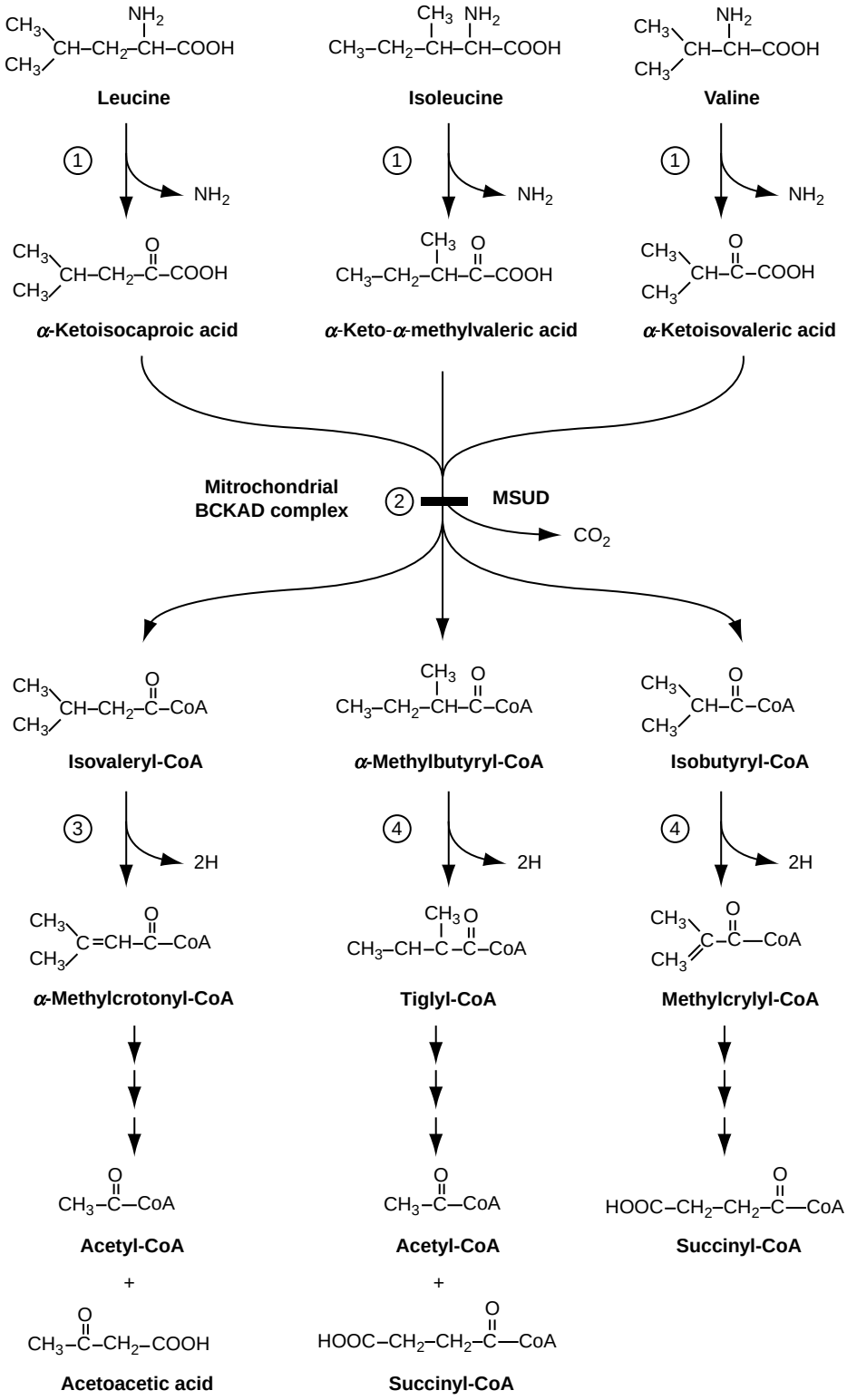



**FIGURE 1** Catabolic pathways for the branched-chain amino acids (BCAAs) leucine, isoleucine, and valine. The first three common reactions are catalyzed by the following enzymes: reversible transamination by BCAA aminotransferases (1), oxidative decarboxylation of BCKAs and esterification of CoASH by the single mitochondrial branched-chain α-ketoacid dehydrogenase (BCKD) complex (2) and dehydrogenation by isovaleryl-CoA dehydrogenase (3) or α-methyl branched-chain acyl-CoA dehydrogenase (4). After these steps the degradation pathway for each amino acid diverges. As end products, leucine yields acetyl-CoA and acetoacetic acid; isoleucine produces acetyl-CoA and succinyl-CoA; and valine is converted exclusively to succinyl-CoA. The solid horizontal bar (center) depicts the metabolic block imposed by Maple Syrup Urine Disease mutations in the BCKD complex.

## Regulation of BCAA and BCKA Oxidation

The oxidation of BCAAs and BCKAs is tightly regulated but exhibits tissue-specific patterns. The plasma concentrations of BCAAs and BCKAs are elevated in starvation and in clinical conditions such as diabetes mellitus, obesity, and MSUD. Paradoxically, BCAA oxidation is accelerated in muscles from fasted and diabetic rats. Epinephrine ($10^{-5}$–$10^{-6}$ M) and glucagon ($2 \times 10^{-8}$–$5 \times 10^{-9}$ M) were shown to also stimulate BCAA oxidation in the heart and hemidiaphragms of rats. Insulin decreases the oxidation of BCKAs in striated muscle of fed rats, whereas the same hormone increases oxidative decarboxylation of the ketoacids during starvation. Clofibrate administration augments BCAA oxidation in muscle, but inhibits their degradation in liver. Carnitine, ketone bodies, hexanoate, and octanoate increase the oxidation of leucine by skeletal muscle, while pyruvate and decanoate exert inhibitory effects. The metabolism of KIC in isolated rat hepatocytes is inhibited by fatty acids, KIV, KMV, and pyruvate. The mechanism of regulation for BCAA and BCKA oxidation was not fully elucidated in the above earlier studies. However, it has now become clear that these controls are through alteration in activity state or the degree of de-phosphorylation of the BCKD complex.

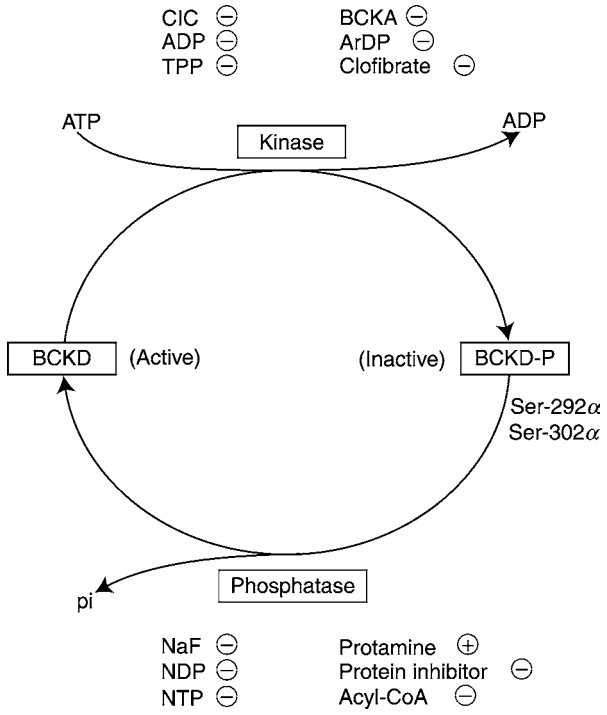



**FIGURE 2** Regulation of the BCKD complex by the phosphorylation–dephosphorylation cycle. Phosphorylation at Ser-292 and Ser-302 of the E1α subunit by BCKD kinase in the presence of ATP results in the inactivation of the BCKD complex. Removal of the phosphate group on E1 converts the BCKD complex back to the active form. Inhibitors (−) and activators (+) for each enzyme are shown. CIC, α-chloroisocaproate; ArDP, arylidene pyruvate; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate.

## The Macromolecular Organization of the BCKD Complex

The mammalian BCKD complex is a member of the highly conserved α-ketoacid dehydrogenase complexes comprising pyruvate dehydrogenase complex (PDC), α-ketoglutarate dehydrogenase complex (α-KGDC), and the BCKD complex with similar structure and function. The mammalian BCKD multi-enzyme complex consists of three catalytic components: a heterotetrameric ($\alpha_2\beta_2$) branched-chain α-ketoacid decarboxylase or E1, a homo-24 meric dihydrolipoyl transacylase or E2, and a homodimeric dihydrolipoamide dehydrogenase or E3. The E1 and E2 components are specific for the BCKD complex, whereas the E3 component is common among the three α-ketoacid dehydrogenase complexes. In addition, the mammalian BCKD complex contains two regulatory enzymes: the specific kinase and the specific phosphatase that regulate activity of the BCKD complex through phosphorylation (inactivation)/dephosphorylation (activation) cycles (Figure 2). The six subunits with their cofactors and prosthetic groups that make up the mammalian BCKD complex are shown in Table I. Three-dimensional structures for the E1 and kinase components and the lipoyl-bearing and subunit-binding domains of the E2 component of the mammalian BCKD complex have been determined. The BCKD complex is organized around a cubic E2 core, to which 12 copies of E1, up to six copies of E3, and unknown numbers of the kinase and the phosphatase are attached through ionic interactions (Figure 3). The molecular mass of the BCKD multienzyme complex is estimated to be $4 \times 10^6$ Da. The purified bovine E1–E2 subcomplex has a sedimentation coefficient ($S_{20,w}$) of 40 S. The E3 component has low affinity for the E2 core, and was mostly lost during purification of the mammalian BCKD complex.

The reaction steps catalyzed by the three enzyme components are also shown in Figure 3. The E1 component catalyzes a thiamin diphosphate (ThDP)-mediated decarboxylation of the α-ketoacids and the subsequent reduction of the lipoyl moiety, which is covalently bound to E2. The reduced lipoyl moiety and the lipoyl domain serve as a "swinging arm" to transfer the acyl group from E1 to CoA giving rise to acyl-CoA. Finally, the E3-component with tightly bound flavin adenine dinucleotide (FAD) reoxidizes the dihydrolipoyl residue of E2 with $NAD^+$ as the ultimate electron

**TABLE I**

**Component Enzymes and Subunit Composition of the Mammalian Branched-Chain α-Ketoacid Dehydrogenase (BCKD) Complex**

| Component | Molecular mass (Da) | Prosthetic group (P) and cofactor (C) |
|---|---|---|
| BCKA decarboxylase (E1) | $1.7 \times 10^5$ ($\alpha_2\beta_2$) | ThDP (C) |
| $\alpha$-Subunit | 46 500 | $Mg^{2+}$ (C) |
| $\beta$-Subunit | 37 200 | $K^+$ |
| Dihydrolipoyl transacylase (E2) | $1.1 \times 10^6$ ($\alpha_{24}$) | Lipoic acid (P) |
| Subunit | 46 518[a] | |
| Dihydrolipoyl dehydrogenase (E3) | $1.1 \times 10^5$ ($\alpha_2$) | FAD (C) |
| Subunit | 55 000 | |
| BCKD kinase | $1.8 \times 10^5$ ($\alpha_4$) | $Mg^{2+}$ (C) |
| Subunit | 43 000 | |
| BCKD phosphatase | $4.6 \times 10^5$ (?) | None |
| Subunit | 33 000 | |

BCKA, branched-chain α-ketoacid; ThDP, thiamin diphosphate.
[a]Calculated from the amino acid composition deduced from a bovine E2 cDNA. The E2-subnit migrates anomalously as a 52 kDa species in SDS-PAGE.

acceptor. The net or overall reaction is the production of a branched-chain acyl-CoA, $CO_2$ and NADH from a α-ketoacid, CoA and $NAD^+$ (see reaction [1]).

## Dietary and Hormonal Regulation of the BCKD Complex

The regulation of BCKA oxidation was shown to be at the BCKD complex step by using inhibitors such as oleate and palmitoyl carnitine. The mammalian BCKD complex is predominantly regulated by the reversible phosphorylation of the Ser-292 and Ser-302 residues in the E1$\alpha$ subunit. The activity state of the BCKD complex in different tissues is the ratio of the actual activity of a partially de-phosphorylated form to the maximal activity of the fully de-phosphorylated form. Thus, the activity state represents the percentage of the total BCKD complex that is active in a given tissue or cell type. Recent studies showed that the activity state of the BCKD complex in human tissues is 40% in heart, 26% in skeletal muscle, 28% in liver, and only 14% in kidney. Rats fed with low-protein diets show a reduction in the activity state, but not the amount, of the BCKD complex in hepatocytes, compared to chow-fed rats. The decrease in activity state inversely correlates with an increase in the amounts of the BCKD kinase protein and mRNA in rat liver. Starvation, exercise, and diabetes stimulate the activity of the BCKD complex in skeletal muscle by increasing the proportion of active or de-phosphorylated enzyme. Recently, it was shown that glucocorticoids cause a marked reduction in the BCKD kinase mRNA level in rat hepatoma cells and rat liver with a concomitant increase in the activity state of the BCKD complex. Glucocorticoids and acidification also significantly increase the activity state of the BCKD complex in pig kidney cells expressing the hormone receptor.

## Brain Neuropathology of BCKAs

The BCAAs, in particular leucine, are rapidly transported into the brain and actively metabolized. It was proposed that a leucine–glutamate cycle which plays an important role in maintaining a steady supply of glutamate, a major excitatory neurotransmitter for inter-neuronal communication. The BCAAs are nitrogen donors for glutamate synthesis in astrocytes, a major site of BCAA transamination. The amino group is transferred to α-ketoglutarate to yield glutamate. This amino acid, in turn, is converted to glutamine. $^{15}N$-labeled BCAA studies showed that at least one-third of the amino groups of brain glutamate are derived from BCAAs. The BCKAs may be released from astrocytes to the extracellular fluid and taken up by neurons. Although neurons can oxidize KIC, it is preferentially reaminated to leucine. The flux of the reverse transamination in rat cortical synaptosomes is several times greater than the rate of nitrogen transfer from leucine to glutamate. This is in contrast to the flux in astrocytes which is mainly for glutamate synthesis. In the leucine oxidative pathway, acetoacetyl-CoA is generated for ketone synthesis or for cleavage to acetyl-CoA to enter the tricarboxylic acid cycle.

In the classic form of MSUD, the excess BCAAs and BCKAs are likely to interfere with the neuronal and astrocytic metabolism. BCKAs accumulated in MSUD compromise brain energy metabolism by blocking the respiratory chain and inhibiting creatine kinase activity. Perfusion by microdialysis with leucine and KIV has created a microenvironment similar to that found in MSUD. The infusion of leucine resulted in an increase of large neutral amino acids in the extra-cellular space, thereby a decreased concentration in neurons. The infusion of KIC caused an 11-fold increase in leucine and two- to threefold increase in other large neutral amino acids in the extracellular space. This pattern is consistent with active transamination of KIC to leucine. These changes could affect biosynthesis of serotonin and catecholamines, and alter the homeostasis of the leucine/glutamate cycle and the glutamate/glutamine cycle in brain.

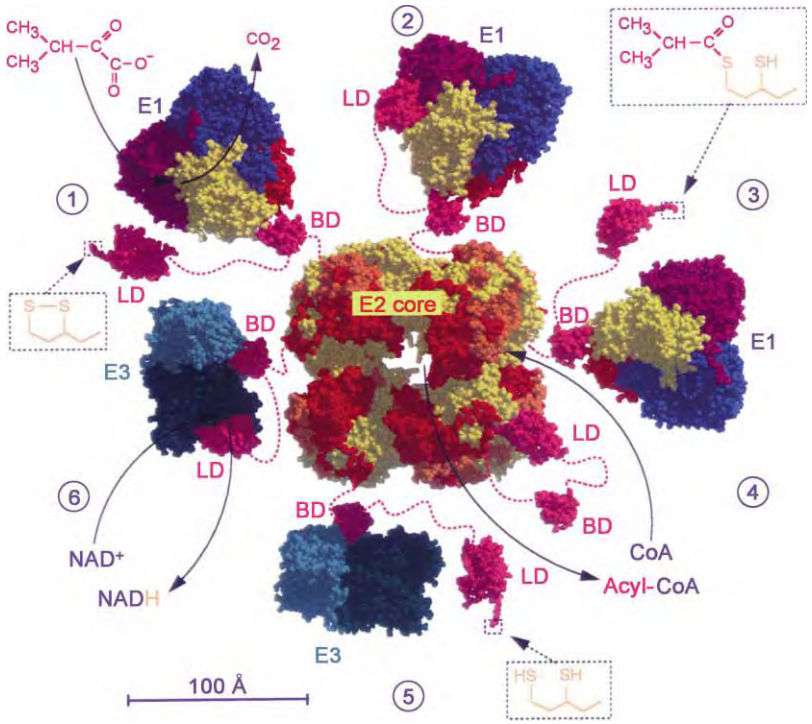



**FIGURE 3** Model for structural organization and individual component reactions of the BCKD complex. The macromolecular structure ($4 \times 10^6$ Da in size) is organized about a cubic transacylase (E2) core (based on the structure of *Azotobacter* pyruvate dehydrogenase E2), to which a decarboxylase (E1) (based upon *Pseudomonas* BCKD E1 structure), a dehydrogenase (E3) (according to the structure of *Azotobacter* pyruvate dehydrogenase E3) are attached through ionic interactions. E2 of the BCKD complex contains 24 identical subunits with each polypeptide made up of three folded domains: lipoyl (LD), E1/E3-binding (BD), and the E2 core domains that are linked by flexible regions (represented by dotted lines). E1 $\alpha_2\beta_2$ heterotetramers or E3 homodimers are attached to BD. The BCKD kinase and BCKD phosphatase (not shown) bind to LD. E1 catalyzes the ThDP-mediated oxidative decarboxylation of α-ketoacids. The ThDP-hydroxyacylidene moiety is transferred to a reduced lipoyl prosthetic group (in the box) on LD. The flexible LD carries S-acyldihydrolipoamide to the active site in the E2 core to generate acyl-CoA. The reduced lipoyl moiety on LD is oxidized by E3 on BD with the concomitant reduction of $NAD^+$. The sum of the above component reactions is the oxidative decarboxylation of BCKAs (reaction [1] in the text).

## See Also the Following Articles

Amino Acid Metabolism • Pyruvate Carboxylation, Transamination and Gluconeogenesis • Pyruvate Dehydrogenase

## Glossary

**activity state** The ratio of the actual (partially de-phosphorylated) activity to the maximal (fully de-phosphorylated) activity of the mammalian mitochondrial branched-chain α-ketoacid (BCKD) complex. The activity state represents % of the total BCKD complex that is active in a tissue under defined physiological conditions.

**ketoacidosis** A clinical condition associated with the accumulation of the branched-chain α-ketoacids derived from branched-chain amino acids leucine, isoleucine and valine present in dietary proteins. This pathological state is presented by patients with the Maple Syrup Urine Disease caused by congenital defects in the BCKD complex.

**oxidative decarboxylation** The removal of a $CO_2$ group from a molecule by a decarboxylase/dehydrogenase with a concomitant transfer of electrons from the substrate to a biological electron acceptor. For the branched-chain α-ketoacids, this reaction is catalyzed by the BCKD complex coupled with the reduction of $NAD^+$.

**phosphorylation** The posttranslational modification of a protein or enzyme molecule by a specific kinase, in which the γ-phosphoryl group from ATP is incorporated into a serine, threonine or tyrosine residue of the protein/enzyme as a means to regulate its biological activity.

**transamination** A reversible enzymatic reaction that converts an amino acid into a corresponding α-ketoacid. For branched-chain amino acids, this reaction is carried out by the distinct isozymes of branched-chain aminotransferase both in the cytoplasm and the mitochondrion.

## Further Reading

Chuang, D. T., and Shih, V. E. (2001). Maple syrup urine disease (Branched-chain ketoaciduria). In *The Metabolic and Molecular Bases of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, eds.) 8th edition, pp. 1971–2006. McGraw-Hill, New York.

Harper, A. E., Miller, R. H., and Block, K. P. (1984). Branched-chain amino acid metabolism. *Annu. Rev. Nutr.* **4**, 409–454.

Harris, R. A., Hawes, J. W., Popou, K. M., Zhao, Y., Shimomura, Y., Sato, J., Jaskiewicz, J., and Hurley, T. D. (1997). Studies on the regulation of the mitochondrial α-ketoacid dehydro-

genase complexes and their kinases. *Adv. Enzyme Regul.* **37**, 271–293.

Perham, R. N. (2000). Swinging arms and swinging domains in multifunctional enzymes: Catalytic machines for multistep reactions. *Annu. Rev. Biochem.* **69**, 961–1004.

Reed, L. J., Damuni, Z., and Merryfield, M. L. (1985). Regulation of mammalian pyruvate and branched-chain α-keto acid dehydrogenase complexes by phosphorylation–dephosphorylation. *Curr. Topics Cell. Regul.* **27**, 41–49.

Yudkoff, M. (1997). Brain metabolism of branched-chain aminoacids. *Glia* **21**, 92–98.

## Biography

David T. Chuang is a professor in the Department of biochemistry at the University of Texas Southwestern Medical Center in Dallas. His principal research interests are in the structure/function and molecular genetics of the human branched-chain α-ketoacid dehydrogenase (BCKD) complex. His laboratory determined three-dimensional structures for components and domains of the human BCKD complex, and characterized mutations in the BCKD genes of patients with the inherited Maple Syrup Urine Disease.

# Brassinosteroids

**Steven D. Clouse**
*North Carolina State University, Raleigh, North Carolina, USA*

**Brassinosteroids (BRs) are endogenous plant growth-promoting hormones that function at low concentrations to influence cellular expansion and proliferation, while interacting with other plant hormones and environmental factors to regulate the overall form and function of the plant. BRs are found throughout the plant kingdom in seeds, pollen, and young vegetative tissue, and the examination of the phenotype of mutants affected in BR biosynthesis or signaling provides genetic evidence that BRs are essential for normal organ elongation, vascular differentiation, male fertility, timing of senescence, leaf development, and responses to light. BRs are unique among plant hormones in their close structural similarity to vertebrate and invertebrate steroid hormones, which have well-known roles in regulating embryonic and postembryonic development and adult homeostasis in mammals and insects.**

## Brassinosteroid Structure and Natural Occurrence

BRs have been identified throughout the plant kingdom as naturally occurring compounds in monocot and dicot angiosperms, gymnosperms, algae, and pteridophytes. At least 40 free BRs and four conjugates have been rigorously characterized from plant tissue by a variety of biochemical approaches.

### Discovery and Chemical Structure of Brassinolide

The discovery of brassinolide, the most active natural BR currently identified, was preceded by three decades of experiments at the United States Department of Agriculture (USDA) in which organic extracts of pollen from over 60 species were applied to a variety of crop plants to identify new compounds with growth-promoting properties. An extract from *Brassica napus* pollen was extremely potent in promoting cell elongation and division in bean second internodes and enhanced overall growth of radishes, leafy vegetables, and potatoes when young seedlings were sprayed in greenhouse experiments. USDA researchers identified the active component of the *B. napus* extract in 1979 and named the novel compound brassinolide. It was determined by single-crystal X-ray analysis to be a polyhydroxylated derivative of 5$\alpha$-cholestane, namely (22*R*,23*R*,24*S*)-2$\alpha$,3$\alpha$,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5$\alpha$-cholestan-6-one (Figure 1).

Other BRs differ from brassinolide by variations at C-2 and C-3, the presence of a ketone or de-oxo function instead of a lactone at C-6, various substitutions at C-24, and the stereochemistry of the hydroxyl groups in the side chain. Known BR conjugates include glycosylated, myristylated, and laurylated derivatives of the hydroxyls at C-2 and C-3 or in the side chain. Many BRs are biosynthetic precursors or metabolic products of brassinolide, although some of these BRs are believed to have independent biological activity in specific plants.

### Endogenous Levels of BRs in the Plant Kingdom

Endogenous levels of BRs vary according to plant organ type, tissue age, and species, with pollen and immature seeds containing the highest levels, followed by young growing shoots. Pollen and immature seeds generally show BR levels of 1–100 ng $g^{-1}$ fw; shoots and leaves typically have lower amounts, 0.01–0.1 ng $g^{-1}$ fw. BRs are isolated from plant tissues by organic solvent extraction followed by reverse-phase high-performance liquid chromatography (HPLC). Accurate quantification of endogenous levels involves spiking with deuterated forms of the BR of interest followed by gas chromatography–mass spectrometry with selected ion monitoring (GC–MS–SIM) analysis of derivatized BR samples.

## Brassinosteroid Biosynthesis

The BR biosynthetic pathway can be divided into general sterol synthesis (cycloartenol to campesterol) and the BR-specific pathway from campesterol to brassinolide. Details of the pathway have been obtained by following the fate of labeled intermediates fed to cell-suspension cultures and whole plants, and by measuring

 © 2004, Elsevier Inc. All Rights Reserved. 

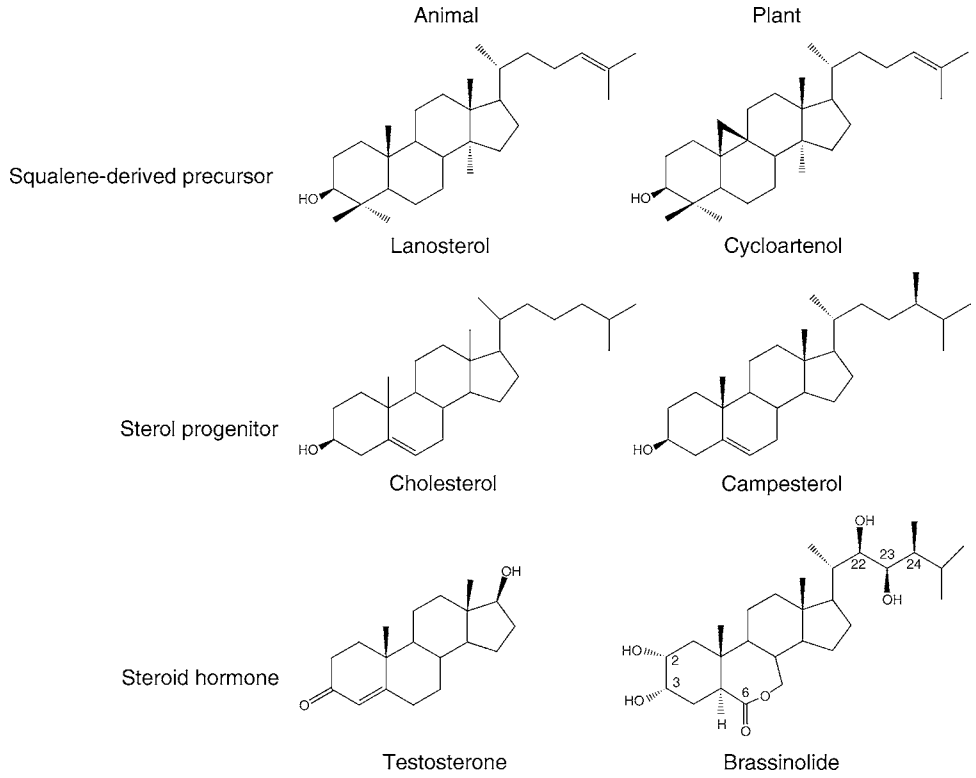



**FIGURE 1** Structure of brassinolide and its sterol progenitor is compared with the animal steroid hormone testosterone and its sterol progenitor.

endogenous sterol and BR levels in biosynthetic mutants blocked at various steps of the pathway.

## Mevalonic Acid to Cycloartenol

Like animal steroids, BRs are products of the isoprenoid biosynthetic pathway beginning with acetyl-CoA and proceeding through intermediates such as mevalonate, isopentenyl pyrophosphate, farnesyl pyrophosphate, and squalene-2,3-epoxide. It appears that the biosynthetic steps from mevalonate to squalene-2,3-epoxide are conserved among the phyla, but in animals and fungi squalene-2,3-epoxide is converted to lanosterol, the precursor of cholesterol, whereas in plants it is converted to cycloartenol, the progenitor of the plant sterols campesterol, stigmasterol, and sitosterol (Figure 1).

## Cycloartenol to Campesterol

The conversion of cycloartenol to campesterol begins with C-24 alkylation of the side chain catalyzed by the enzyme sterol methyl transferase 1 (SMT1) in the presence of S-adenosylmethionine. The product, 24-methylenecycloartenol, is converted to 24-methylenelophenol in a series of steps including a C-14 sterol reductase and a $\Delta^7$–$\Delta^8$ sterol isomerase, encoded in *Arabidopsis* by the *FACKEL* and *HYDRA1* genes, respectively. Mutants in the *SMT1*, *FACKEL*, and *HYDRA1* genes show dwarfism and severe defects in embryogenesis and vascular development that are not rescued by BR treatment, possibly because the early sterol pathway may produce a non-BR signaling molecule that is critical for these developmental processes.

The conversion of 24-methylenelophenol to campesterol involves a $\Delta^7$-C-5-desaturase, encoded by *DWARF7* (*DWF7*); a $\Delta^7$-sterol reductase, encoded by *DWARF5* (*DWF5*); and a C-24 sterol reductase, encoded by *DWARF1* (*DWF1*). *Arabidopsis dwf7*, *dwf5*, and *dwf1* mutants are intermediate dwarfs with altered vascular development and have reduced BR levels. These developmental defects can be rescued by treatment with exogenous BRs.

## Campesterol to Brassinolide

Four reactions lead from campesterol to campestanol via reduction of the C-5 double bond (Figure 2). One of these steps requires a 5$\alpha$-steroid reductase (encoded by the *DE-ETIOLATED2*, *DET2*, gene) that is an orthologue of the mammalian enzyme that catalyzes the NADPH-dependent reduction of testosterone to dihydrotestosterone. From campestanol, two biosynthetic routes are possible: early C-6 oxidation, in which a ketone is introduced at C-6 before the hydroxylation of the side chain, and late C-6 oxidation, in which the side chain is

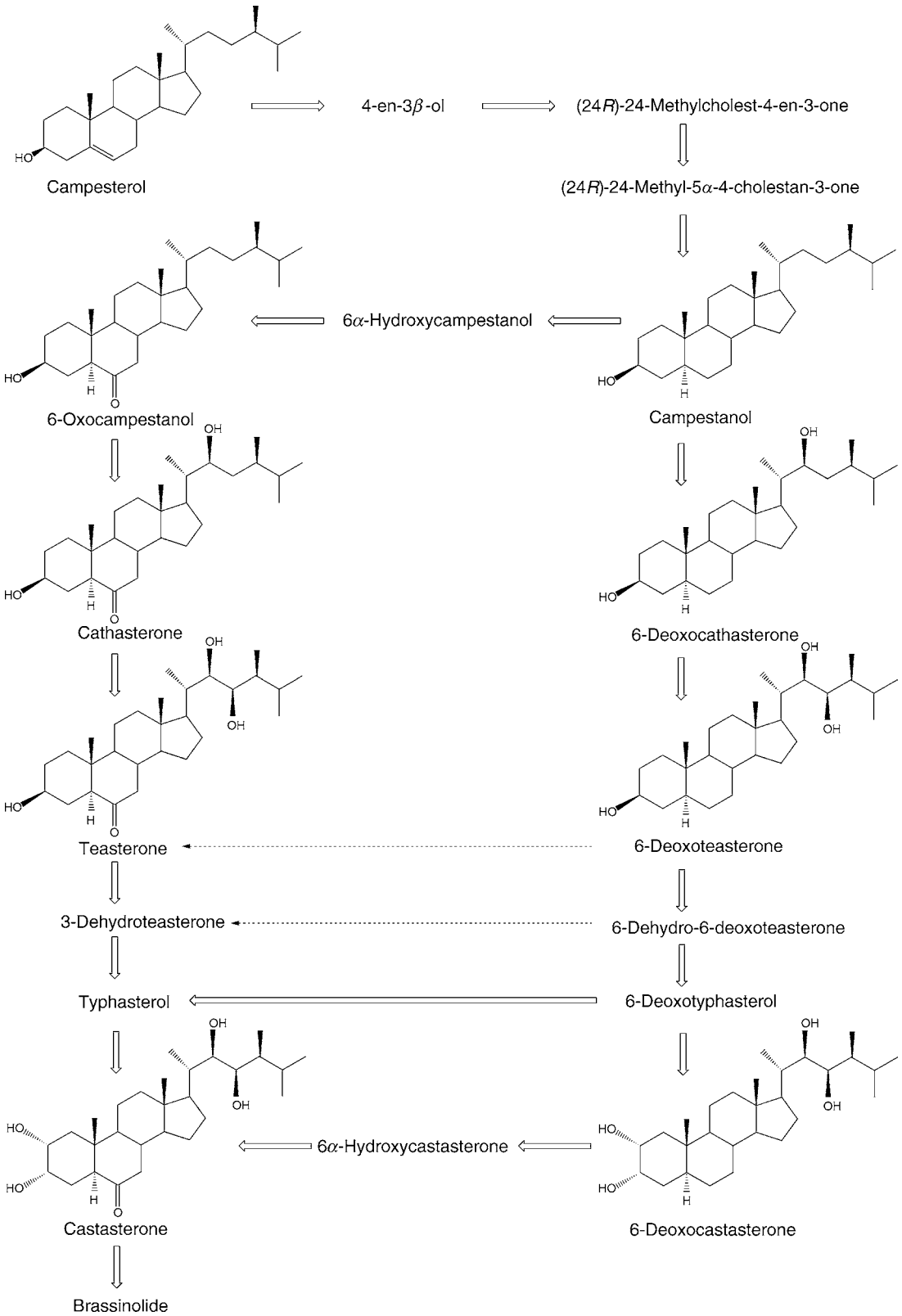



**FIGURE 2** Biosynthetic pathway leading from the plant sterol campesterol to brassinolide. From campestanol, two alternative pathways exist, early C-6 oxidation (left) and late C-6 oxidation (right). Several points of interconnection are possible between the two pathways.

modified first and the ketone is introduced in the penultimate step. Many plants, including *Arabidopsis*, have both pathways functional, whereas others, such as tomato, appear to use only late C-6 oxidation. Hydroxylation of the side chain in either pathway occurs via successive steps involving the cytochrome P-450 steroid hydroxylases DWARF4 (DWF4) and CONSTITUTIVE PHOTOMORPHISM AND DWARFISM (CPD).

The *det2*, *dwf4*, and *cpd* mutants of *Arabidopsis* are extremely dwarf in stature; have dark-green, rounded, and downward-curling leaves; and exhibit a prolonged life span with reduced fertility and altered vascular development. In the dark, these mutants show some of the features of light-grown plants, including shortened hypocotyls and open cotyledons. All these phenotypic alterations can be rescued to wild type by exogenous application of brassinolide or BR intermediates downstream of the biosynthetic block caused by the mutant.

## Brassinosteroid Physiology

BRs have a dramatic positive effect on stem elongation, promoting epicotyl, hypocotyl, and peduncle elongation in dicots and enhancing coleoptile and mesocotyl growth in monocots. Exogenous BRs also stimulate tracheary element differentiation and numerous studies *in vivo* suggest that endogenous BRs are essential for normal vascular development. In many systems, BRs increase rates of cell division, particularly under conditions of limiting auxin and cytokinin. BRs also promote seed germination, accelerate senescence, cause hyperpolarization of membranes, stimulate ATPase activity, and alter the orientation of cortical microtubules. In addition to effects on growth, BRs also mediate the effects of abiotic and biotic stresses, including salt and drought stress, temperature extremes, and pathogen attack.

## Brassinosteroid Signal Transduction

Recently it has been shown that animal steroids can be recognized by cell-surface receptors. However, the classic signaling pathway for these hormones involves binding the steroid to an intracellular receptor consisting of a variable N-terminal domain, a highly conserved DNA-binding domain with two zinc-fingers, and a multifunctional domain that mediates ligand-binding, dimerization, and ligand-dependent transcriptional activation. The completed genome sequence of *Arabidopsis thaliana* indicates that plants do not contain members of this superfamily of intracellular steroid receptors, suggesting that cell-surface recognition is the primary, if not only, form of plant steroid perception. Thus, plant and animal steroid hormones share many structural and functional features, but differ in their primary signaling pathways.

### Receptor Kinases and BR Perception

The best characterized component of BR signal transduction is *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*), a single genetic locus in *Arabidopsis* encoding a leucine-rich repeat receptor kinase. Homologous genes have been identified in rice, tomato, and pea. Numerous *bri1* mutant alleles have been identified by various genetic screens, most of which exhibit the extreme dwarfism and other phenotypic characteristics of severe BR-deficient mutants. In contrast to the biosynthetic mutants, *bri1* mutants cannot be rescued by BR treatment, consistent with their role in signal transduction.

The BRI1 protein consists of a putative signal peptide followed by the extracellular domain proper including a leucine zipper and 25 leucine-rich repeats that are flanked by short sequences containing paired cysteines. A 70-amino-acid island that is critical for biological function is embedded between repeats 21 and 22. Downstream of the extracellular domain lies a short hydrophobic single-pass transmembrane domain, followed by the juxtamembrane region and the cytoplasmic Ser-Thr kinase domain. Binding studies with radiolabeled brassinolide and microsomal fractions of wild-type, mutant, and transgenic *Arabidopsis* plants clearly shows that BRI1 is an essential component of the BR receptor complex. Whether it binds BR directly or in association with other sterol-binding proteins is currently unclear.

The mechanism of action of many animal receptor kinases involves ligand-mediated homo- or heterodimerization of the receptor followed by autophosphorylation of the intracellular kinase domain. This activation of the kinase results in the recognition and phosphorylation of downstream signaling components, leading ultimately to the regulation of specific gene expression. Plant receptor kinases appear to follow the same general paradigm of receptor kinase action, and mutational analysis shows that both a functional extracellular domain and an active cytoplasmic kinase are essential for BRI1 function.

With respect to BRI1 and BR signaling, recent evidence suggests that heterodimerization with another leucine-rich repeat receptor kinase may play an important role. BAK1 (BRI1 associated receptor kinase 1) shares similar structural organization to BRI except that it has only five leucine-rich repeats in its extracellular domain and lacks the 70-amino-acid island of BRI1. BAK1 is expressed in all tissues of the plant, similar to the global expression pattern of BRI1, and confocal laser

microscopy of transgenic plants expressing fusion proteins shows plasma membrane localization for both BRI1 and BAK1. Direct physical interaction between BRI1 and BAK1 was confirmed in yeast cells and *Arabidopsis* plants by co-immunoprecipitation experiments and genetic studies strongly suggest that BAK1 plays an important role in BR signaling.

## Downstream Components

Both BRI1 and BAK1 have been shown to possess kinase activity *in vitro*, and they can autophosphorylate themselves and transphosphorylate one another. Other *in vitro* substrates of the BRI1 kinase have been identified, but no *in vivo* cytoplasmic substrates of either BRI1 or BAK1 have been characterized thus far. An element further downstream from BRI1/BAK1 is the BIN2 (brassinosteroid insensitive 2) kinase, a negative regulator of BR signal transduction. BIN2, which is homologous to insect and mammalian shaggy-like kinases involved in the Wingless/Wnt signaling pathways, most likely acts by phosphorylating two related cytoplasmic proteins, BZR1 and BES1. Phosphorylation by BIN2 targets BZR1 and BES1 for proteasome-mediated degradation. In the presence of BR, BIN2 activity is apparently inhibited and the nonphosphorylated forms of BZR1 and BES1 accumulate and move to the nucleus where they interact with unknown transcription factors to regulate the expression of specific genes involved in the BR response. Our current knowledge of BR signal transduction is summarized in Figure 3.

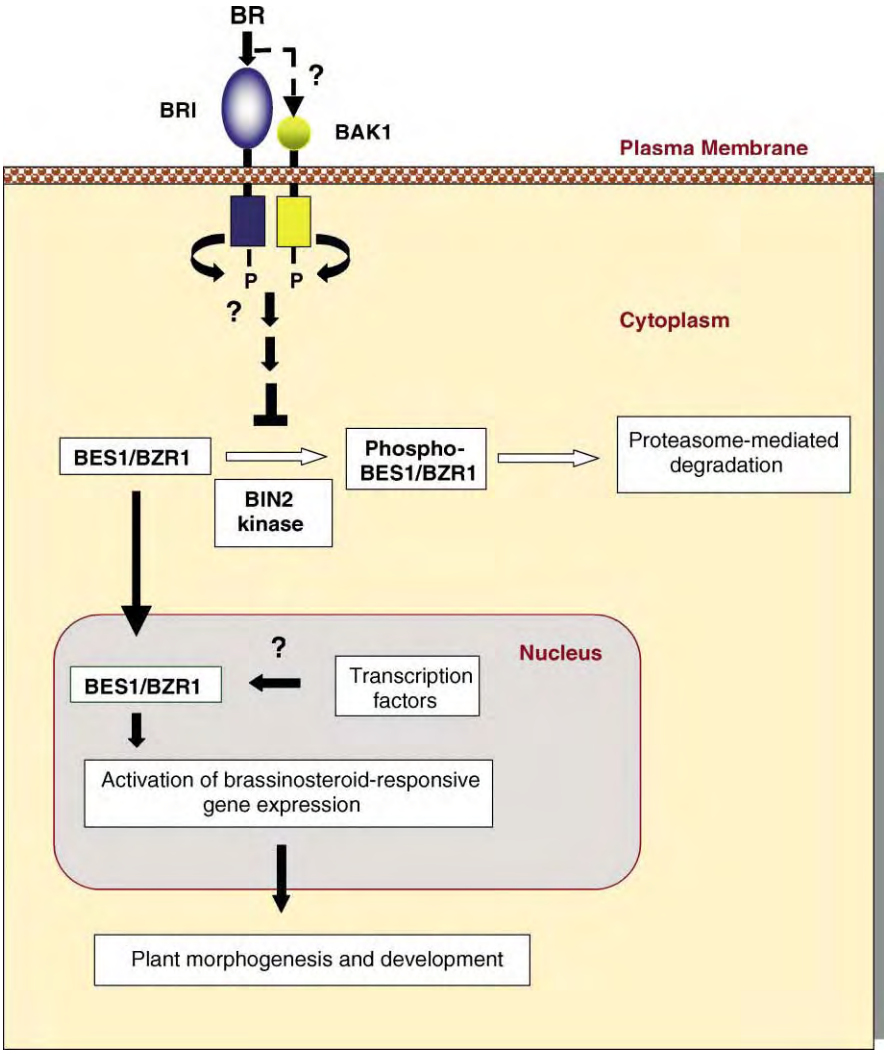



**FIGURE 3** A model for BR signal transduction in *Arabidopsis*. BR binds to BRI1 or the BRI1/BAK1 heterodimer and initiates a signaling cascade that inactivates the BIN2 kinase. This allows accumulation and nuclear localization of the unphosphorylated forms of the positive regulators BES1 and BZR1. Question marks indicate proposed but uncharacterized steps. Adapted from *Molecular Cell*, Vol. 10, S. D. Clouse, Brassinosteroid Signal Transduction, pp. 973–982, Copyright 2002, with permission from Elsevier.

## See Also the Following Articles

Cholesterol Synthesis • G Protein-Coupled Receptor Kinases and Arrestins

## Glossary

**brassinolide** The most active brassinosteroid. It affects cell elongation and division at nanomolar levels. A unique polyhydroxylated steroid with a C-6, C-7 lactone.

**gas chromatography–mass spectrometry with selected ion monitoring (GC–MS–SIM)** Technique used with deuterated standards to determine endogenous levels of brassinosteroids in plants.

**receptor kinase** A signal-transduction protein with multiple domains, including an extracellular ligand-binding domain, a membrane-anchoring region, and an intracellular kinase. Numerous receptor kinases occur in various cell types in both plants and animals.

## Further Reading

Altmann, T. (1999). Molecular physiology of brassinosteroids revealed by the analysis of mutants. *Planta* **208**, 1–11.

Bishop, G. J., and Koncz, C. (2002). Brassinosteroids and plant steroid hormone signaling. *Plant Cell* **14**, S97–S110.

Clouse, S. D. (2002). Brassinosteroid signal transduction: Clarifying the pathway from ligand perception to gene expression. *Mol. Cell* **10**, 973–982.

Clouse, S. D., and Sasse, J. M. (1998). Brassinosteroids: Essential regulators of plant growth and development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 427–451.

Sakurai, A., Yokota, T., and Clouse, S. D. (eds.) (1999). *Brassinosteroids: Steroidal Plant Hormones*. Springer, Tokyo.

## Biography

Steven D. Clouse is a Professor in the Department of Horticultural Science at North Carolina State University in Raleigh. His principal research interests are brassinosteroid signal transduction and the regulation of gene expression by plant steroids. He holds a Ph.D. from the University of California, Davis and received his postdoctoral training at the Salk Institute for Biological Studies in La Jolla, California. His group cloned some of the first BR-regulated genes and was the first to identify the *bri*1 mutant, which is the focus of much current BR signal transduction research.

# Cadherin Signaling

**David B. Sacks and Jonathan M.G. Higgins**
*Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA*

**The associations of cells with one another regulate many cellular processes. Adherens junctions are specialized adhesive structures between cells that are formed by cadherin-dependent interactions. Cadherins are a family of $Ca^{2+}$-dependent intercellular adhesion molecules linked to the cytoskeleton. Cadherins are also signaling molecules that convey information from the environment to the interior of the cell. In addition, signals from inside the cell can modulate adhesion through cadherins. These forms of cadherin signaling, together with their ability to support adhesion, allow cadherins to participate in coordinating many aspects of cellular organization.**

## The Cadherin Family

The cadherin superfamily includes six main subfamilies. These are type-I (or classical) cadherins, type-II (atypical) cadherins, desmocollins, desmogleins, protocadherins, and Flamingo cadherins.

### Classical and Atypical Cadherins

Classical cadherins like E-, N-, and P-cadherin are components of adherens junctions between cells. The most highly characterized is E-cadherin, which is predominantly expressed in epithelium. The classical cadherins are single-span transmembrane proteins with a highly conserved carboxy-terminal cytoplasmic domain and five extracellular domains, termed cadherin repeats. $Ca^{2+}$ binding to the extracellular domain induces homophilic adhesion with cadherin molecules on adjacent cells. The cytoplasmic tail of cadherin associates with the actin cytoskeleton via proteins called catenins. Cadherin binds $\beta$-catenin or $\gamma$-catenin (also termed plakoglobin), which in turn bind to $\alpha$-catenin (Figure 1). The interaction of $\alpha$-catenin with the actin cytoskeleton strengthens the intercellular adherens junction. Another catenin, termed $p120^{ctn}$, also binds to the cadherin tail in the cytoplasm. The closely related atypical cadherins have similar properties.

### Desmosomal Cadherins

Desmocollins and desmogleins are components of desmosomes, which are sites of cell–cell adhesion in tissues subjected to mechanical strain (e.g., epidermis and the myocardium). The overall structure of these desmosomal cadherins resembles that of the classical cadherins, but their cytoplasmic tails are linked to the intermediate filaments of the cytoskeleton.

### Protocadherins

The protocadherin family members have as many as seven extracellular $Ca^{2+}$-binding domains, a transmembrane region and divergent cytoplasmic domains. They appear to participate in development, and may have a particularly important role in the central nervous system.

### Other Cadherins

Other cadherins range from those with seven transmembrane segments (such as Flamingo) to T-cadherin, which lacks both transmembrane and cytoplasmic regions.

## Functions of Cadherin Signaling

Originally described exclusively as cell adhesion proteins, cadherins have been shown to influence multiple aspects of cell behavior. Indeed, cadherins can transmit signals across the cellular membrane into the interior to modulate cell function. While many members of the cadherin family are likely to share this capacity, to date only members of the classical and atypical cadherin families have been studied in detail and they will be the focus of this article.

### Signaling Triggered by Cell–Cell Contact

Signaling can be triggered when cadherins attach to other cadherins on adjacent cells, allowing information about the external environment to be conveyed into the cell. For example, most normal cells grown in culture dishes will reproduce until they form a single continuous layer. A process known as "contact inhibition" then prevents further cell division. Cadherin–cadherin junctions

 © 2004, Elsevier Inc. All Rights Reserved. 

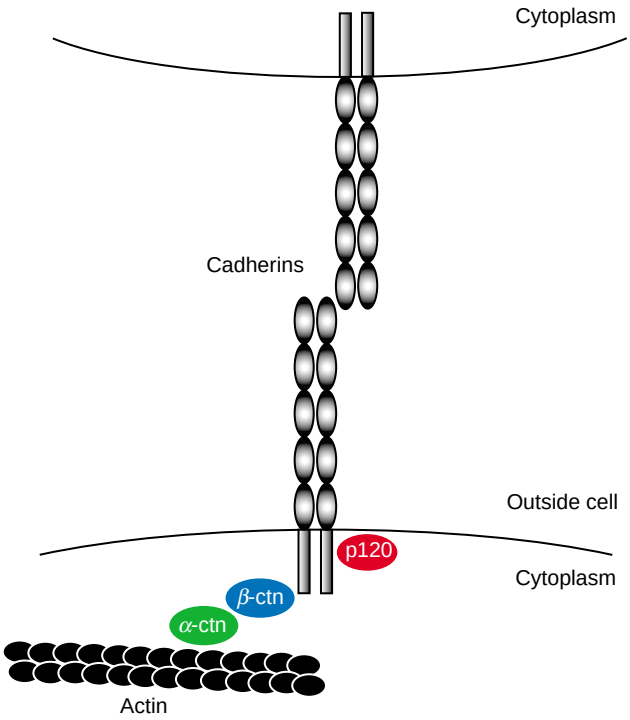



**FIGURE 1** Schematic model of a cadherin-mediated adherens junction. Cadherins on adjacent cells interact with one another to provide intercellular adhesion. The terminal portion of the cytoplasmic tail of cadherin binds to β-catenin (β-ctn) or γ-catenin (not shown), which in turn binds to α-catenin (α-ctn). The interaction of α-catenin with the actin cytoskeleton stabilizes the adherens junction. Another catenin, $p120^{ctn}$ (p120), interacts with the cytoplasmic portion of the cadherin tail near the cell membrane.

between cells produce a signal that leads to contact inhibition and renders the cells insensitive to the stimulatory effects of growth factors. Signals generated by interactions between cadherins are also implicated in the formation of desmosomal cell junctions, nerve outgrowth, establishment of cell polarity, cytoskeletal organization, tissue formation, cell differentiation, cell movement, and regulation of gene expression.

### Influence of Cadherin Level on Signaling

The amount of cadherin present on the cell surface may influence the signals generated. For example, expression of high levels of E-cadherin can inhibit cell motility and cell division and induce cell polarity. These effects have led to the description of E-cadherin as a tumor suppressor. In fact, loss of functional E-cadherin is often associated with epithelial tumor progression and invasion. Regulation of E-cadherin gene transcription alters the total amount of E-cadherin protein in cells during morphogenic processes such as hair follicle development. The level of E-cadherin on the cell surface also can be specifically regulated. These changes are likely to modulate not only the adhesive properties of the cell, but also the signals that the cell receives through contacts with other cells. In some cases, alterations in the level of cadherin may regulate cellular function even in the absence of cadherin–cadherin interactions between cells.

### Different Signaling Properties of Various Cadherins

It is notable that the different types of cadherins induce different changes in cell behavior. For example, while E-cadherin usually reduces the motility of a cell, the presence of N-cadherin or cadherin-11 has the opposite effect, enhancing the ability of cells to crawl over surfaces and invade tissue. It is clear that the various cadherins trigger different signaling pathways. These differences are likely to play a critical role in the regulated changes in cell behavior that occur during early animal development ("embryogenesis"). A striking example of this sort of change occurs when cells undergo "epithelial–mesenchymal transitions." This process occurs during tissue remodeling when relatively sedentary epithelial cells with stable adherens junctions transform into more motile and invasive mesenchymal cells in order to migrate and form new tissue structures. These events are frequently accompanied by a switch in cadherin expression, for example, from E-cadherin to N-cadherin. Similar changes occur during malignant transformation. Most human cancers arise from epithelial cells. These cells normally express E-cadherin. In many cases, expression of functional E-cadherin is lost during malignant transformation, and anomalous expression of N-cadherin or cadherin-11 occurs. These changes may contribute to the increased growth rate, and increased migratory and invasive capacity of cancer cells.

### Inside-Out Cadherin Signaling

It is also apparent that the adhesiveness of cell surface cadherins can be modulated from within the cell in the absence of changes in the level of cadherin expression. Therefore, cadherins are able to transduce both "outside-in" and "inside-out" signals. Feedback between these two types of signals is critical to allow the correct formation and disassembly of cadherin–cadherin junctions during morphogenesis and for the continuous remodeling of adherens junctions that occurs even in sedentary cells.

## Mechanisms of Cadherin Signaling

Considerable progress has been made in describing cadherin signaling. Classical and atypical cadherins

interact with several signaling molecules, but the molecular mechanisms that constitute the signaling pathways are not completely understood. Three major signaling pathways have been described.

## Rho Family GTPase Signaling

An important target of cadherin signaling is the cytoskeleton. This dynamic scaffold of rod-like actin must be rearranged to allow cells to form junctions with other cells and for cells to move. Members of the Rho family of small GTPases are vital regulators of these events, and they are activated in response to the formation of cadherin-mediated cell junctions. Changes in the activity of the Rho family GTPases also lead to alterations in the adhesive capacity of cadherins. Thus, communication between cadherin and Rho GTPases is bidirectional. In general, GTPases serve as switches that are "on" when bound to GTP and "off" when the bound GTP is hydrolyzed to GDP. The best-characterized members of the Rho family are Cdc42, Rac1, and RhoA.

Cdc42 induces the formation of filopodia or microspikes, small finger-like projections from the cell that are needed for the first steps of adherens junction formation by cadherins. Cdc42 is activated upon the assembly of E-cadherin-mediated cell junctions (Figure 2). This mechanism perhaps allows E-cadherin to initiate signals that in turn further stimulate adherens junction formation. Cdc42 also activates the so-called PAR/atypical protein kinase (aPKC) complex and thereby stimulates cell polarity and tight junction formation.

Rac1 is activated rapidly in response to cadherin binding. Active Rac1 stimulates remodeling of the actin cytoskeleton that leads to the formation of cell membrane protrusions known as lamellipodia. This change in morphology may help extend the contact zone between cells, enhancing formation of stable adherens junctions. Activated Cdc42 or Rac1 can also influence the function of a protein known as IQGAP1. High levels of IQGAP1 in a cell decrease E-cadherin-mediated adhesion, perhaps by binding $\beta$-catenin, thus displacing $\alpha$-catenin from the E-cadherin–$\beta$-catenin complex.

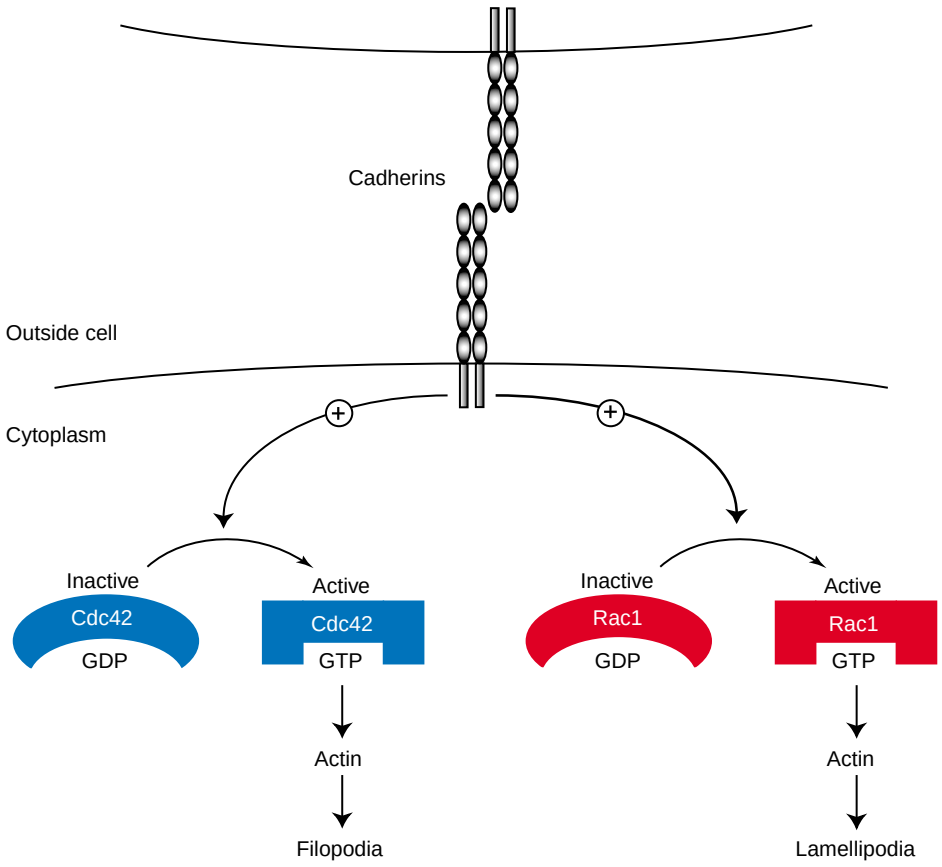



**FIGURE 2** Cadherin-mediated adhesion activates Rho family GTPases. The formation of cadherin contacts between cells leads to the activation of Cdc42 and Rac1 (i.e., generation of GTP-bound forms). In the active state, Cdc42 and Rac1 cause alterations in the actin cytoskeleton that lead to the generation of finger-like filopodia and membrane ruffles known as lamellipodia, respectively. These events lead to changes in both the ability of cells to adhere to their surroundings and in cell migration, and can increase the stability of the cadherin-mediated junctions themselves.

Activated Cdc42 or Rac1 can bind to IQGAP1 and counteract this activity, thereby increasing the adhesive capacity of cadherins.

Activation of RhoA leads to the formation of stress fibers. Active RhoA influences E-cadherin function and appears necessary for maintenance of adherens junctions. Moreover, cadherin signals can modulate the activity of RhoA. Thus, cadherin can regulate the actin cytoskeleton by multiple mechanisms.

The transition from the off to the on state of GTPases can be brought about by guanine nucleotide exchange factors (GEFs) that cause the displacement of GDP from the GTPase, and replacement with GTP. Conversely, GTPase activating proteins (GAPs) enhance the conversion of GTP to GDP, so inactivating the GTPase. It is likely that cadherin-mediated adhesion leads to the activation of GEFs, or perhaps the inactivation of GAPs, for Cdc42 and Rac1. RhoA may be regulated by direct interactions with $p120^{ctn}$.

## Wnt/β-Catenin Signaling

In addition to linking the cytoplasmic tails of cadherins to the actin cytoskeleton (see Figure 1), β-catenin is an important component of a signaling pathway involved in both normal development and cancer. Normally, β-catenin molecules not attached to cadherins are degraded in the cytoplasm. When cells are exposed to an extracellular growth factor known as Wnt, β-catenin is protected from degradation and accumulates in the nucleus (Figure 3). There, in association with the TCF/LEF family of DNA-binding proteins, β-catenin activates the transcription of genes that stimulate cell proliferation. Thus, the extent to which β-catenin is "soaked up" by binding to

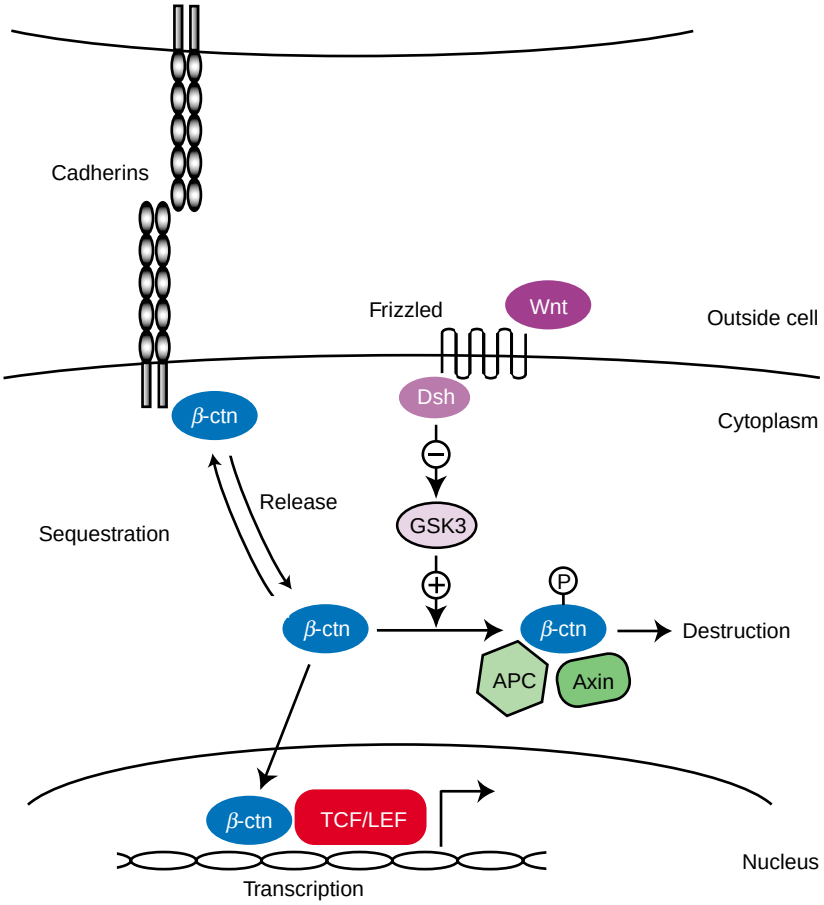



**FIGURE 3** Cadherins modulate Wnt/β-catenin signaling. In addition to its role as a component of adherens junctions, β-catenin (β-ctn) increases the activity of the TCF/LEF transcription factor in the nucleus. The extent to which β-catenin accumulates in the nucleus depends in part upon the level of free β-catenin in the cytoplasm. The amount of free β-catenin is normally low due to its phosphorylation by glycogen synthase kinase-3 (GSK3), binding to adenomatous polyposis coli (APC) and Axin proteins, and subsequent destruction (P indicates the added phosphate group). GSK3 activity is decreased by Disheveled (Dsh) when the growth factor Wnt binds to its receptor frizzled on the cell surface. Therefore, Wnt can reduce degradation of β-catenin and increase the amount of β-catenin in the nucleus, thereby promoting transcription. In contrast, the formation of cadherin–cadherin junctions, or an increase in the amount of cadherin, can lead to the sequestration of β-catenin due to its interaction with cadherin tails. In this way, cadherins are thought to decrease the amount of nuclear β-catenin, and act in opposition to Wnt signals.

cadherins can potentially modulate the signal generated by activation of the Wnt pathway. High expression levels of E-cadherin can substantially reduce the amount of free β-catenin, and lower the cellular response to Wnt. In the absence of Wnt, increased adherens junction formation recruits β-catenin, decreasing the nuclear pool of β-catenin, and attenuating β-catenin-mediated transcription. Conversely, disruption of cell–cell adhesion may release β-catenin, thereby enhancing transcription. It is noteworthy that mutations of proteins that regulate turnover of β-catenin have been found in several human cancers.

## Phosphorylation and Dephosphorylation

The phosphorylation of proteins (i.e., addition of phosphate groups) by kinase enzymes is a common mechanism for regulating numerous cellular processes. Although the cadherins themselves do not have kinase activity, a number of examples of cross talk have been observed between cadherins and growth factor receptors that contain intrinsic tyrosine kinases (Figure 4). For example, N-cadherin interacts with the fibroblast growth factor receptor FGFR1 and can boost the phosphorylation cascade triggered by binding of fibroblast growth factor-2 to FGFR1. Analogous interactions may occur between E-cadherin and the epidermal growth factor receptor, as well as VE-cadherin and the vascular endothelial growth factor receptor-2. In this way, cadherins can modulate changes in the survival, proliferation and motility of cells in response to a variety of growth factors.

Conversely, phosphorylation can modulate inside-out signals to regulate cadherin activity. The association between growth factor receptor tyrosine kinases and cadherins allows growth factors to promote the phosphorylation of several adherens junction proteins (Figure 4). A number of non-receptor kinases, including Src, also can phosphorylate junction components. Phosphorylation of β-catenin on tyrosine residues is believed to induce dissociation of β-catenin from cadherins and decrease adherens junction stability. Phosphorylation of the cadherins themselves, and of γ-catenin and $p120^{ctn}$, also modulates adherens junction assembly. In particular, the interaction of $p120^{ctn}$ with the cytoplasmic domain of cadherins appears to be an important regulator of cadherin activity, perhaps by influencing the clustering of cadherins in the cell membrane. Cadherins are also found in a complex with selected protein phosphatases. These enzymes, which are activated by cadherins, dephosphorylate (i.e., remove phosphate from) β-catenin, allowing reversible regulation of cadherin adhesive capacity (Figure 4).

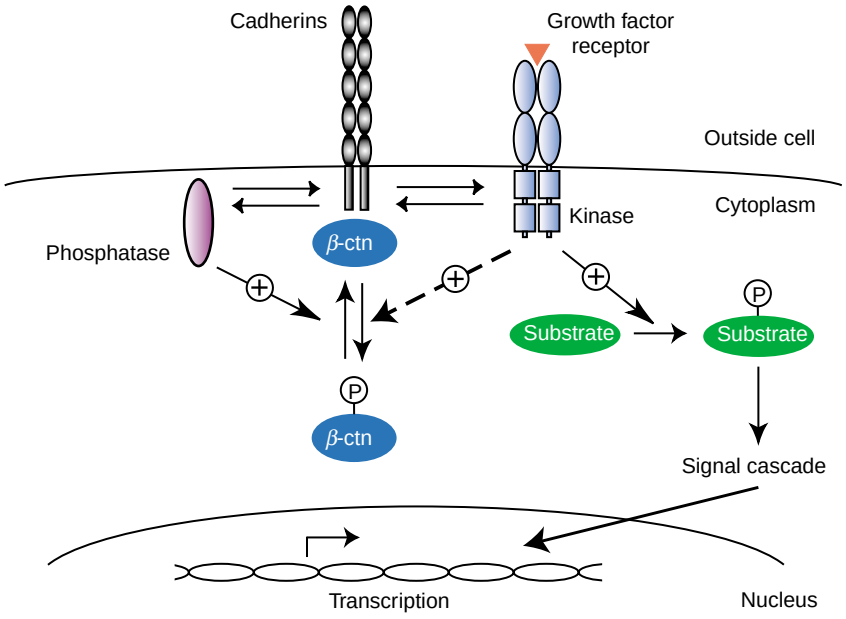



**FIGURE 4** Cadherin crosstalk with kinases and phosphatases. Cadherins interact with a number of growth factor receptors that have intrinsic kinase activity. By this mechanism, cadherins can modulate the signaling cascades triggered by growth factors that lead, for example, to the activation of gene transcription. In addition, activation of receptor kinases can promote the phosphorylation on tyrosine residues of β-catenin (dotted line; P indicates the added phosphate group), its dissociation from cadherin tails, and disassembly of the adherens junction (not shown). Cadherins are also found in a complex with a number of protein phosphatases that can dephosphorylate β-catenin. In this way, cadherins can modulate the reversible phosphorylation of adherens junction components that alter the activity of cadherin itself.

## See Also the Following Articles

Cadherin-Mediated Cell–Cell Adhesion • Rho GTPases and Actin Cytoskeleton Dynamics

## Glossary

**adherens junction** Cadherin-dependent adhesive structures linked to the actin cytoskeleton that mediate attachment of cells to one another.

**cadherins** Family of homophilic adhesion molecules that mediate $Ca^{2+}$-dependent cell–cell adhesion.

**catenins** Family of proteins comprising $\alpha$-, $\beta$-, $\gamma$-, and p120 catenin, that attach to the cytoplasmic tails of cadherins.

**homophilic** The preference of a molecule for interactions with identical molecules.

**Rho family GTPases** Family of regulatory proteins that act as molecular switches, alternating between active and inactive forms.

## Further Reading

Ben-Ze'ev, A., Shtutman, M., and Zhurinsky, J. (2000). The integration of cell adhesion with gene expression: The role of beta-catenin. *Exp. Cell Res.* **261**, 75–82.

Bracke, M. E., Van Roy, F. M., and Mareel, M. M. (1996). The E-cadherin/catenin complex in invasion and metastasis. *Curr. Top Microbiol. Immunol.* **213**, 123–161.

Braga, V. M. (2002). Cell–cell adhesion and signalling. *Curr. Opin. Cell Biol.* **14**, 546–556.

Christofori, G., and Semb, H. (1999). The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem. Sci.* **24**, 73–76.

Gumbiner, B. M. (2000). Regulation of cadherin adhesive activity. *J. Cell Biol.* **148**, 399–403.

Perez-Moreno, M., Jamora, C., and Fuchs, E. (2003). Sticky business: Orchestrating cellular signals at adherens junctions. *Cell* **112**, 535–548.

Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev.* **14**, 1837–1851.

## Biography

David B. Sacks is an Associate Professor in the Department of Pathology at Brigham and Women's Hospital and Harvard Medical School. His principal research interest is in the field of signal transduction, with a focus on $Ca^{2+}$ and calmodulin signaling. He holds an M.B.Ch.B from the University of Cape Town (South Africa) and received his postdoctoral training at Washington University.

Jonathan M. G. Higgins is an Assistant Professor in the Division of Rheumatology, Immunology, and Allergy at Brigham and Women's Hospital, Harvard Medical School. He holds a D.Phil. from the University of Oxford.

# Cadherin-Mediated Cell–Cell Adhesion

**Frauke Drees and W. James Nelson**
*Stanford University, Stanford, California, USA*

**Cell–cell adhesion is the process of establishment and maintenance of contacts between adjacent cells in tissues and organs of multicellular organisms. Cadherins comprise a superfamily of transmembrane glycoproteins that mediate calcium-dependent cell–cell adhesion and are found in all metazoans. Cadherins are involved in a wide variety of biological processes including development, tissue morphogenesis, and tumor metastasis. The cadherin family consists of four major subfamilies: classical cadherins, desmosomal cadherins, protocadherins, and atypical cadherins. Members of the cadherin superfamily share a conserved extracellular, calcium-binding domain (CD repeat) involved in specifying cell–cell adhesion, but the number (5–34) and arrangement of these domains differ between subfamily members.**

## Cadherin Subfamilies

### Classical Cadherins

Classical cadherins comprise a small subfamily of proteins with the simplest, prototypic organization of cadherins: an extracellular domain comprising five EC repeats, a single transmembrane domain, and a conserved cytoplasmic domain that binds specific cytosolic proteins that link cadherins to the actin cytoskeleton. They are also the best understood of all cadherins in terms of structure, mechanism of adhesion, and function. Generally, classical cadherins are expressed in specific tissues, from which they were originally named (e.g., epithelia, E-cadherin; nervous system, N-cadherin; placenta, P-cadherin) although it is now recognized that each is broadly expressed in most tissues. Classical cadherins have been implicated in a variety of developmental processes, particularly in cell sorting during tissue remodeling. They are the major components of adherens junctions, an ultrastructurally defined adhesion site between opposing cells; note that other adhesion proteins such as nectin, a member of the immunoglobulin superfamily of adhesion proteins, have also been shown to localize and contribute to the formation and maintenance of adherens junctions. Classical cadherins mostly form homotypic adhesions between the same type of cadherin on adjacent cells (e.g., E–E, N–N, P–P).

### Desmosomal Cadherins

Desmosomal cadherins, termed desmocollin and desmoglein, are found in desmosomes, a structure morphologically distinct from the adherens junction. Desmosomal cadherins have five EC domains, a single transmembrane domain, and a cytoplasmic domain that interacts with cytosolic proteins different from those that bind classical cadherins that link desmosomal cadherins to the intermediate filament network. Desmosomes and desmosomal cadherins are typically found in tissues that have to withstand high mechanical stress, such as epithelia, and are well conserved throughout most higher metazoans, but are absent in flies and worms. There are three subtypes of desmocollins and desmogleins, which are expressed in a tissue- and differentiation-specific manner. Desmosomal cadherins form heterotypic interactions, in contrast to the homotypic interactions of classical cadherins. Desmosomal cadherins can initiate and maintain cell–cell adhesion in the absence of classical cadherins, but adherens junctions containing classical cadherins are generally assembled before desmosomes in development.

### Protocadherins

Protocadherins constitute the largest cadherin subfamily, but its members are far less well characterized than either classical or desmosomal cadherins. Protocadherins are highly expressed in the mammalian brain and nervous system. Genomically, they are organized in three large gene clusters with a very large number of exons encoding "variable" extracellular domains, and three exons encoding "constant" intracellular domains; combinations of extracellular and intracellular domains can be assembled to potentially generate thousands of different protocadherins. This has prompted speculation that protocadherin genes might reorganize in a manner

 © 2004, Elsevier Inc. All Rights Reserved. 

similar to that of immunoglobulins (hence the analogy to immunoglobulin "variable" and "constant" domains), and their resulting diversity might contribute to specifying cell–cell connections during development of the nervous system. This is substantiated by the fact that protocadherins show distinct spatio-temporal expression patterns during brain development. However, a clear role for protocadherins in cell–cell adhesion has not yet been demonstrated.

### Atypical Cadherins

Atypical cadherins comprise the fat-like cadherins (e.g., fat, dachsous, two related proteins expressed in *Drosophila*), cadherins with a seven-pass transmembrane domain (e.g., flamingo), protein kinase cadherins, Dcad102F-like cadherins, and T-cadherin. Fat-like cadherins are a heterogeneous subfamily of proteins with very large extracellular domains that contain up to 34 EC repeats, in addition to EGF and laminin-G domains. They have a single transmembrane domain, and the cytoplasmic domains of fat and dachsous have predicted binding sites for cytoplasmic proteins that bind classical cadherins. The seven-pass transmembrane cadherins, such as flamingo, were first identified in *Drosophila*. The extracellular domain contains eight or nine EC domains and EGFR- and laminin-like domains, and mediates homotypic cell–cell adhesion. Amino acid sequence analysis predicts seven transmembrane domains, which show similarity to G protein-coupled receptors (GPCR). The cytoplasmic domain is different from those of classical and fat-like cadherins, and interacting cytosolic proteins have not been found, but due to their similarity to GPCR, flamingo-like cadherins have been implicated in cell signaling pathways. In *Drosophila*, flamingo-like cadherins are required for establishment of planar cell polarity. T-cadherin differs from all other cadherins because it lacks transmembrane or cytosplasmic domains, but instead associates with the membrane via a glycophosphidylinositol-anchor that inserts directly into the outer leaflet of the plasma membrane lipid bilayer. T-cadherin can mediate calcium-dependent cell–cell adhesion, but does not cluster in cell–cell contacts. T-cadherin has been implicated in signaling events and might constitute a negative guidance cue for neurons in the nervous system. Little is known about the other members of this subfamily, protein kinase cadherins, and Dcad102F-like cadherins.

## Regulation of Cadherin Adhesion: Role of the Extracellular Domain

Although it is broadly accepted that the main role of cadherins is to mediate adhesion between cells, the exact mechanism of how the adhesive contact forms is not fully resolved. Analysis of this problem has focused primarily on the classical cadherins: N- and E-cadherin. Classical cadherins are believed to form two types of dimers, lateral or *cis*-dimers between two cadherin molecules on the same cell, and *trans*-dimers between cadherins on opposite cells (Figure 1).

### Mechanisms of Cadherin Specificity in Cell Sorting

Specificity of adhesion appears to be determined in two ways. Early cell-sorting experiments showed that when two cell lines expressing N- and E-cadherin were mixed the cells sorted out and formed separate aggregates each of which expressed the same cadherin. In this case, homotypic *trans* adhesion between extracellular domains of the same cadherins appears to mediate adhesion specificity. The specificity for homotypic recognition is thought to be encoded in the EC1 extracellular domain, because when the EC1 domain of N-cadherin is swapped onto EC domains 2–5 of E-cadherin for example, the resulting chimeric protein sorts like N-cadherin in the experiments described earlier. More recent experiments showed that cells also sort from each other in aggregates formed between cells expressing different levels of the same cadherin. Thus, cell sorting may also be mediated by differences in adhesion strength that is proportional to the amount of cadherin expressed. The exact mechanism involved in adhesion specificity remains to be elucidated, but it will probably be a combination of spatio-temporal expression patterns of different cadherins, as well as differences in adhesive properties (strengths) between different cadherins.

### Extracellular Domain Structure (*cis*- and *trans*-Dimers)

These early studies indicated that the outermost, N-terminal cadherin domain (EC1) is important in adhesion of E- and N-cadherin, and that a specific tripeptide, histidine–alanine–valine (HAV), and a conserved trytophan residue at position 2 (W2) in EC1 formed cadherin-binding motifs. Short peptides comprising the HAV sequence block adhesion in cell aggregation assays, and mutations of W2 also decrease adhesion. Both the EC1 domain and the EC1–EC2 domain of E- and N-cadherin have been crystallized. The crystal structures reveal that EC domains comprise an immunoglobulin-like fold with a seven-strand $\beta$-sheet, and that 6 $Ca^{2+}$-ions associate with residues in the linker region between EC1 and EC2. Calcium binding stabilizes the extracellular domain into a rigid bent rod-like structure that is resistant to proteolytic

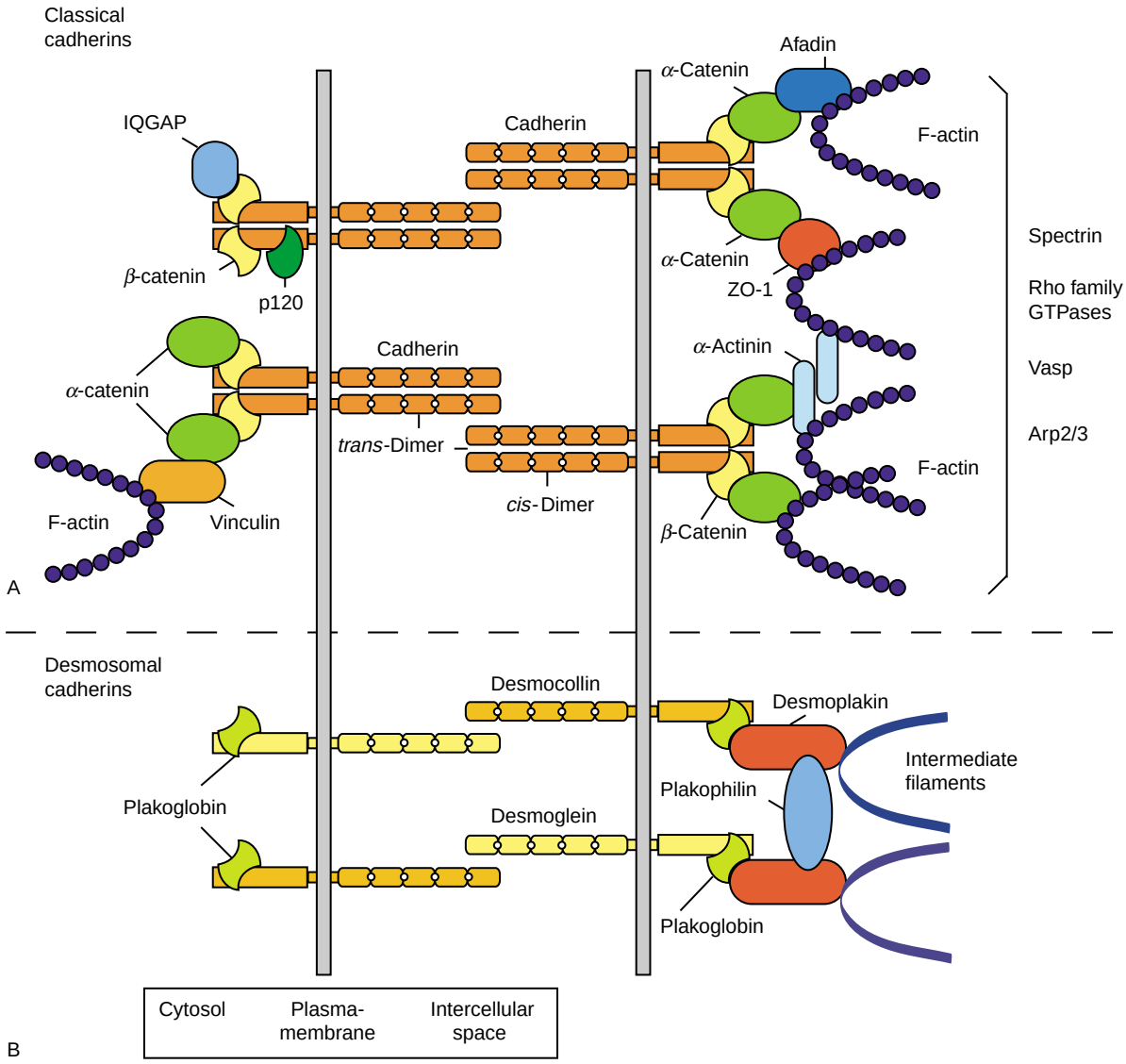



**FIGURE 1** Schematic model of the linkage of protein–protein interactions between cadherins and cytoplasmic proteins. (A) Classical cadherins form *cis*- and *trans*-dimers to mediate $Ca^{2+}$-dependent adhesion. The cytoplasmic domain interacts directly with p120 and β-catenin. β-catenin in turn binds α-catenin, which links the complex to the actin cytoskeleton both directly through interaction with actin filaments and indirectly through the actin-binding proteins vinculin, ZO-1, α-actinin, and afadin. Other actin-associated proteins such as Rho-family GTPases, Vasp, Arp2/3, and spectrin have been shown to localize at adherens junctions, but the molecular nature of their interactions is less well defined. IQGAP binds β-catenin and has been suggested to negatively regulate adhesion by causing α-catenin to dissociate from β-catenin. (B) The desmosomal cadherins, desmocollin and desmoglein, form heterodimers and are linked to the intermediate filament network through interaction with plakoglobin, desmoplakin, and plakophilin.

cleavage, which may explain why cadherin adhesion is dependent on extracellular $Ca^{2+}$. Several models have been proposed for *cis*- and *trans*-dimerization. Mechanisms involved in *cis*-dimerization are poorly understood. Early models favored *trans*-dimerization through interaction between two opposing EC1 domains, but biophysical studies argue for *trans*-interaction involving contact between several EC domains. Further analysis of cadherin extracellular domains and their interactions will be required to resolve the mechanism of interaction and adhesion.

## Regulation of Cadherin Adhesion: Role of the Cytoplasmic Domain

### Linkage To β-Catenin Family Members

Classical and desmosomal cadherins are linked to the cytoskeleton via their intracellular domains through a class of cytosolic proteins collectively called catenins: β-catenin, plakoglobin (γ-catenin), and p120 (Figure 1). There are different binding sites on the cadherin

cytoplasmic domain for p120 catenin, and β-catenin and plakoglobin. p120 interacts directly with the juxtamembrane region of the cadherin intracellular domain, but its role in adhesion is not clearly defined; it may negatively modify adhesive strength of cadherin interaction, or regulate cadherin clustering. β-catenin and plakoglobin bind a conserved C-terminal domain of classical cadherins in a mutually exclusive manner and, in turn, interact with α-catenin to link the complex to the actin cytoskeleton (Figure 1). Desmosomal cadherins also bind plakoglobin, but not β-catenin; plakoglobin in turn binds cytosolic proteins termed plakophillin and desmoplakin that link desmosomal cadherins to the intermediate filament cytoskeleton (Figure 1). How different catenins specifically bind to different members of the cadherin superfamily is poorly understood, but it may be dependent on structural recognition between different β-catenin family members and the cytoplasmic domain of different cadherin subtypes.

### Linkage to the Cytoskeleton

When cells initiate cell–cell contact, additional proteins are rapidly recruited to the cadherin-β-catenin/plakoglobin-α-catenin complex, which results in clustering of cadherins and strengthening of cell–cell adhesion. α-catenin binds directly to actin filaments, but also interacts with several actin-binding proteins including α-actinin, vinculin, ZO-1, and afadin (Figure 1). Afadin also binds the transmembrane adhesion protein nectin, which is localized with cadherins to the adherens junction, thereby interlinking the cadherin and nectin adhesion complexes. Other proteins such as VASP, IQGAP, fodrin, and members of the Rho family of small GTPases are localized to cadherin-based adhesion junctions and these proteins may further regulate actin assembly and organization at sites on the plasma membrane involved in cadherin-mediated adhesion (Figure 1). The importance of these cytosolic proteins to cell–cell adhesion is illustrated by the fact that α-catenin-deficient tumor cell lines or cell lines expressing E-cadherin lacking the intracellular domain adhere very poorly despite the expression of E-cadherin and other adherens junction proteins. Reintroduction of α-catenin causes these cells to form fully developed adhesion junctions.

### Regulation by Phosphorylation

Cadherin complex assembly and disassembly is a highly regulated process. Both cadherin and β-catenin can be phosphorylated on serine/threonine and tyrosine residues, which modifies their binding affinity for each other. Serine/threonine phosphorylation by casein kinase II increases the binding affinities between cadherin/β-catenin and β-catenin/α-catenin, thereby stabilizing the cadherin/catenin complex and strengthening adhesion. Conversely, tyrosine-phosphorylation of either cadherin or β-catenin appears to destabilize the adhesion complex and weaken adhesion.

### Functions in Signaling

In addition to the role as linkers between cadherins and the cytoskeleton, β-catenin, plakoglobin and p120 function as co-activators of gene transcription with members of the LEF/Tcf family of transcription factors in the Wnt/*wingless* pathway. Whether functions of β-catenin in the cytoplasm (cell–cell adhesion) and nucleus (gene expression) are interchangeable or separate is not known.

## Roles of Cadherins in Cell and Tissue Morphogenesis

Classical cadherins are essential for embryonic development and have long been implicated in cell sorting, and in tissue formation and integrity. As noted above, two cell populations expressing different types, or protein levels of cadherins form separate aggregates when mixed together, and in development cell populations sort from one another by the same mechanism. To obtain evidence of morphogenic roles of cadherin in development, several cadherin genes have been deleted (Table I). E-cadherin knockout mice exhibit the most severe phenotype with very early lethality due to inability of pre-implantation embryos to fully compact and to develop into blastocysts. The N-cadherin knockout results in embryonic death at embryonic days 9–10 due to heart defects and malformation of the neural tube. In contrast, P-cadherin knockout mice are viable and fertile with only slight abnormalities in mammary glands. This demonstrates that while specific cadherins are important in development and morphogensis, some cadherins can be substituted by another. While the phenotype of the E- and N-cadherin knockout mice can be explained by adhesion defects, it is an open question as to whether there are defects in induction of tissue differentiation due to lack of a specific cadherin. In this context it is noteworthy that embryonic stem (ES) cells derived from E-cadherin −/− embryos form different tissues depending upon which cadherin is used to rescue cell–cell adhesion. When injected subcutaneously into mice, wild-type ES cells form benign teratomas that are highly differentiated and show a variety of tissue subtypes. By contrast, the E-cadherin null ES cells formed teratomas with no organized tissue structures. Interestingly, null cells rescued with overexpressed E-cadherin formed teratomas of almost exclusively epithelia, whereas overexpression of N-cadherin in the

**TABLE I**

**Effects of Gene Deletions of Selected Cadherins and Adherens Junction Components on Mouse Development**

| Gene/protein | Subfamily | Genetic defect | Phenotype |
|---|---|---|---|
| E-cadherin | Classical cadherin | Knockout, deletion of extracellular $Ca^{2+}$-binding and adhesion motif | Embryonic lethal at time of implantation, embryonic compaction occurs, probably due to maternally deposited E-cadherin, but embryos fail to form trophectoderm or blastocyst cavity. |
| N-cadherin | Classical cadherin | Knockout | Embryonic lethal E9–10, major heart defects and malformed neural tube and somites. |
| P-cadherin | Classical cadherin | Knockout | Viable and fertile, despite high expression of P-cadherin in placenta. Virgin P-cadherin-null females exhibit precocious differentiation of mammary gland. Hyperplasia and dysplasia of mammary epithelium with age. |
| R-cadherin | Classical cadherin | Knockout | Viable and fertile, dilated proximal kidney tubules, defects in development of ureteric bud- and metanephric-mesenchymal-derived cells during nephrogenesis. |
| Cadherin-6 | Classical cadherin | Targeted gene disruption (deletion of membrane targeting sequence) | Viable and fertile, transition of fraction of mesenchymal aggregates into epithelial structures in nephrogenesis is delayed. |
| $\beta$-Catenin | AJ component | Knockout | Embryonic lethal E7–9, at day 7 cells detach from ectodermal cell layer, the three germlayers and amniotic folds fail to form, and epithelial organization of ectoderm is completely lost. |
| Desmocollin Dsc1 | Desmosomal cadherin | Knockout | Normal at birth, epidermal fragility and barrier defects, hyperproliferation and abnormal differentiation of epidermis, hair loss, hair follicle degeneration, mice develop ulcerating lesions resembling chronic dermatitis. |
| Desmoglein Dsg3 | Desmosomal cadherin | Knockout | Normal at birth, disintegration of epidermis (Acantholysis), hair loss, runting (probably due to oral lesions). Phenotype resembles pemphigus vulgaris (caused by autoantibodies against Dsg3). |
| Desmoplakin | Desmosome component | Knockout | Embryonic lethal E6.5 due to defects in extra-embryonic tissues and failure of egg-cylinder expansion, abnormal desmosomes. Animals rescued with wild-type chimeras in extra-embryonic tissue die at E10 with major defects in heart muscle, neuro-, and skin epithelium. |
| Plakoglobin | Desmosome component | Knockout | Embryonic lethal E10.5–15 due to heart failure. Reduction in number and size of desmosomes and abnormal structure. Some genetic backgrounds delayed embryonic lethality, epidermal blistering. |

ES cells led to the formation of neuroepithelia and cartilage in the teratomas.

A crucial and reoccurring step during development and tissue morphogenesis is the role of cell–cell adhesion in the structural and functional polarization of cells. One example is the development of polarized epithelial cells. Cadherin-mediated cell–cell adhesion between simple epithelial cells generates a cell monolayer that separates two biological compartments and regulates homeostasis by vectorial transport of ions and solutes between those compartments. This function requires cells to generate and maintain two functionally and structurally distinct plasma membrane domains, termed apical and basal-lateral, which face these different compartments. Cell–cell adhesion initiates structural and biochemical asymmetries at the cell membrane, through assembly of cytoskeleton and vesicle docking complexes that initiate formation of the (basal-) lateral membrane domain. Studies in *Drosophila* have shown that cadherins play additional roles in establishing planar polarity of ommatidia in the eye and wing hairs (fat-like cadherins), and a role for cadherins in migration has been proposed in oocyte border cell migration.

## Consequences of Disruption of Cadherin Functions: Disease States and Cancer

Loss of cell–cell adhesion, changes in cytoskeletal organization, and aberrant adhesion-mediated signaling are hallmarks of malignant transformation, tumors, and cancer. Disruption of cell–cell adhesion might contribute to increased proliferation and migration of tumor cells, thereby leading to invasion of surrounding tissue and metastasis. Alterations of cadherin-mediated cell–cell adhesion arise mainly through three mechanisms: loss of adhesion complex function, most commonly through inactivation of E-cadherin or $\alpha$-catenin expression or function; aberrant cadherin function due to "cadherin switching"; and changes in cell signaling through cadherins or catenins (Table I).

First, inactivating mutations in the E-cadherin gene CDH1 are frequently found in certain carcinomas such as gastric or breast lobular carcinomas, and germ-line mutations in CDH1 strongly predispose individuals to gastric cancer. Loss of E-cadherin expression requires inactivation of both CDH1 alleles, as would be expected for a tumor suppressor gene, and reintroduction of E-cadherin into tumor cell lines can reverse transformation from an invasive to a benign, epithelial tumor cell phenotype in culture. In addition to genetic mutation or gene deletion, down-regulation of E-cadherin at the transcriptional level has been observed in certain tumors, for example through DNA hypermethylation of the CDH1 promoter and/or up-regulation of transcriptional repressors of E-cadherin.

Second, up-regulation of nonepithelial cadherins, such as N-cadherin and cadherin-11, either with or without loss of E-cadherin, may contribute to the transformation and invasiveness of tumor cells. This change in cadherin expression pattern is termed "cadherin switch" and has been found in primary tumors. In tissue cancer cell lines, it causes an epithelial to fibroblast-like transition of cellular phenotype.

Third, alterations in cell signaling pathways through cadherins or catenins contribute to cancer formation. Mutations in $\beta$-catenin generally inhibit $\beta$-catenin degradation and thereby inappropriately activate the Wnt signaling pathway. In addition, tyrosine phosphorylation of E-cadherin and $\beta$-catenin destabilizes the adhesion complex and, therefore, changes in this signaling pathway, for example by activation of tyrosine kinases, might contribute to tumor cell invasiveness by decreasing the amount of cell–cell adhesion.

Mutations in desmosomal cadherins and other protein components of desmosomes, such as plakoglobin or plakophilin, give rise to specific diseases of the skin and, in some cases, heart defects, thereby illustrating the importance of desmosomal adhesion in tissues exposed to high mechanical stress. Patients with the skin conditions Darier–White and Hailey–Hailey disease suffer from defects in keratinocyte adhesion due to breakdown of desmosomal adhesion. In the blistering diseases pemphigus vulgaris and pemphigus foliaceus, which affect skin and mucous cell membranes, auto-immune antibodies against desmoglein 1 and 3, respectively, interact with the extracellular domain of these cadherins, thereby functionally blocking adhesion and leading to the disassembly of desmosomes in the skin.

In summary, the cadherin superfamily of adhesion proteins plays important roles in development, and cellular reorganization and polarization, and abnormalities in cadherins and associated proteins are characteristic of disease states and cancer. While cadherins are known to regulate calcium-dependent cell–cell adhesion, and many associated proteins that link cadherins to the cytoskeleton have been identified, there remain many unanswered questions about the way cadherins form cell–cell contacts, regulate assembly of the cytoskeleton, and determine cell differentiation.

### See Also the Following Articles

Actin Assembly/Disassembly • Actin-Related Proteins • Cadherin Signaling • Rho GTPases and Actin Cytoskeleton Dynamics

## Glossary

**adaptor proteins** Cytosolic proteins that link protein complexes together and allow for regulation of physiological responses. In the example of cell adhesion proteins, adaptor proteins (catenins) provide the linkage between cell adhesion transmembrane proteins and the cytoskeleton.

**cell–cell adhesion** Process of establishment and maintenance of contacts between adjacent cells in tissues and organs of multicellular organisms.

**cell–cell adhesion molecules** Transmembrane glycoproteins that mediate cell–cell binding. There are three major protein families: selectins, immunoglobulin superfamily, and cadherins.

**cell–cell junctions** Specialized regions on the cell surface by which cells are joined to each other. Tight junctions in epithelial cell layers form a ribbon-like seal between compartments. Gap junctions are protein-lined channels between two cells that allow diffusion of small molecules from one cell to the next. Adherens junctions and desmosomes are dense protein plaques connected to cytoskeletal networks that link two adjacent cells together.

**cytoskeleton** Network of fibrous proteins found in the cytosol of eukaryotic cells that provides structural support for the cell, determines cell shape and motility, and allows directional movement of organelles and vesicles inside the cell. There are three major classes of cytoskeletal filaments: actin microfilaments, microtubules, and intermediate filaments.

## Further Reading

Braga, V. M. M. (2002). Cell–cell adhesion and signaling. *Curr. Opin. Cell Biol.* **14**, 546–556.

Fukata, M., and Kaibuchi, K. (2001). Rho-family GTPases in cadherin-mediated cell–cell adhesion. *Nat. Rev. Mol. Cell Biol.* **2**, 887–897.

Hajra, K. M., and Fearon, E. R. (2002). Cadherin and catenin alterations in human cancer. *Genes, Chromosomes Cancer* **34**, 255–268.

Jamora, C., and Fuchs, E. (2002). Intercellular adhesion, signaling and the cytoskeleton. *Nat. Cell Biol.* **4**, 101–108.

Koch, A. W., Bozic, D., Pertz, O., and Engel, J. (1999). Homophilic adhesion by cadherins. *Curr. Opin. Struct. Biol.* **9**, 275–281.

Steinberg, M. S. (1996). Adhesion in development: An historical overview. *Dev. Biol.* **180**, 377–388.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* **7**, 619–627.

Tepass, U., Truong, K., Godt, D., Ikura, M., and Pfeifer, S. (2000). Cadherins in embryonic and neural morphogenesis. *Nat. Rev. Mol. Cell Biol.* **1**, 91–100.

Vleminckx, K., and Kemler, R. (1999). Cadherins and tissue formation: integrating adhesion and signaling. *BioEssays* **21**, 211–220.

## Biography

W. James Nelson is the Rudy J. and Daphne Donohue Munzer Professor in the School of Medicine, Professor of Molecular and Cellular Physiology, and Biological Sciences (by courtesy). Professor Nelson's research focuses on basic cellular mechanisms involved in generating cell asymmetry, particularly in epithelia and neurons. His research has uncovered a hierarchy of cellular events initiated by cell–cell adhesion that leads to cellular remodeling and the generation of structurally and functionally polarized cells.

# Calcitonin Gene-Related Peptide and Adrenomedullin Receptors

**Debbie L. Hay, Alex C. Conner and David R. Poyner**
*Aston University, Birmingham, UK*

**Calcitonin gene-related peptide (CGRP) and adrenomedullin are abundant, related peptides that share many biological actions. Most prominently, both are very potent vasodilators. CGRP is a neurotransmitter in sensory neurons and adrenomedullin is a hormone that is locally released from tissues including vascular endothelium and smooth muscle. Together with calcitonin and amylin, they form a peptide family. For some time, there was considerable controversy about the nature of the receptors for this family. It has been established that they define a new paradigm among G protein coupled receptors (GPCRs), in that they are heterodimers of a seven-transmembrane-spanning protein that resembles a classical GPCR and a member of the single-transmembrane-spanning receptor activity-modifying protein (RAMP) family. Although there is still much to be learned, this discovery has clarified the pharmacology of these peptides and may have implications for other GPCRs.**

## The Pharmacology of CGRP and Adrenomedullin

### The Structure of CGRP and Adrenomedullin

The primary sequences of CGRP and adrenomedullin (Figure 1) share a low level of homology but the secondary structure of these peptides is conserved. For both peptides, an amino-terminal ring is important for receptor activation; peptides that lack this structure ($CGRP_{8-37}$ and $adrenomedullin_{22-52}$) are antagonists.

### The Pharmacology of Endogenous CGRP and Adrenomedullin Receptors

#### CGRP Receptor-Selective Drugs and their Pharmacological Properties

There are few high-affinity, selective drugs that can be used to define CGRP and adrenomedullin receptors. For CGRP, the only widely available antagonist is $CGRP_{8-37}$. This has only limited selectivity. A number of high-affinity nonpeptide antagonists have been developed. The most interesting of these appears to be BIBN4096BS (Figure 2), which shows marked selectivity for primate over rodent CGRP receptors and has very little affinity for non-CGRP receptors. Largely on the basis of work with $CGRP_{8-37}$, it has been suggested that CGRP receptors should be divided into two subtypes. $CGRP_1$ receptors have a high affinity for $CGRP_{8-37}$ ($pA_2 > 7$); $CGRP_2$ receptors have a lower affinity for this antagonist. A number of linear CGRP analogues have been suggested to be $CGRP_1$-selective agonists, but their usefulness has been questioned.

The $CGRP_1/CGRP_2$ classification has proved controversial. $CGRP_{8-37}$ reveals marked heterogeneity in receptors that respond to CGRP in assays of physiological function. However, this is not apparent in radioligand-binding studies. The heterogeneity may reflect the existence of bona fide receptor subtypes, it may be due to cross-reactivity of CGRP at receptors for other peptides, or it may be caused by other factors,

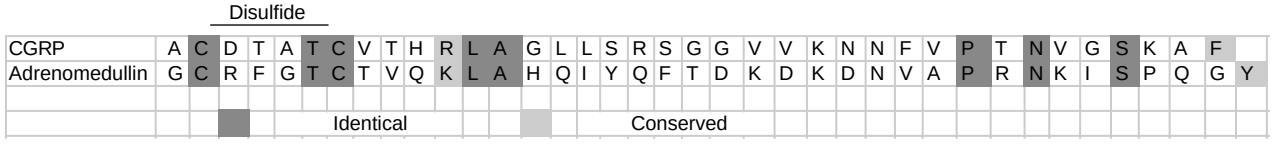



**FIGURE 1** A comparison of the structure of human CGRP and $adrenomedullin_{15-52}$. Note the disulfide bond in both peptides, indicated by a bar over the sequences. The first 14 amino acids of adrenomedullin are not required for biological activity and are not shown.

 © 2004, Elsevier Inc. All Rights Reserved. 

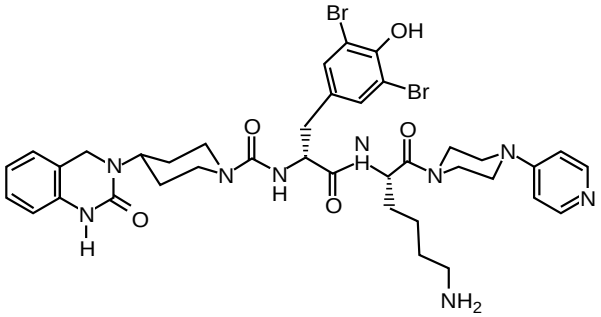



**FIGURE 2** The structure of BIBN4096BS.

such as differential peptidase activity. The issue is likely to be resolved with progress in the molecular characterization of CGRP receptors hand-in-hand with the development of CGRP antagonists.

#### Adrenomedullin-Selective Drugs and their Pharmacological Properties

The only adrenomedullin antagonist that has been widely used is adrenomedullin$_{22-52}$. This is not particularly potent ($pA_2 \sim 7$) and does not antagonize all adrenomedullin-mediated responses. Nonetheless, it has proved useful in some circumstances in defining adrenomedullin receptors.

## The Molecular Structure of CGRP and Adrenomedullin Receptors

### CRLR and RAMPs

CGRP and adrenomedullin receptors display a very unusual molecular architecture. Both are composed of a common seven-transmembrane-spanning protein called calcitonin receptor-like receptor (CRLR or CL; Figure 3). This is a member of the B-family of GPCRs (related to other receptors, such as those for secretin and calcitonin). Human CRLR is 461 amino acids long and has 55.5% sequence identity with the human calcitonin receptor. It has three potential glycosylation sites, although only two appear to be utilized in the mature protein. By itself, CRLR does not function as a receptor for any peptide. However, when associated in a dimer with a RAMP, it functions as a high-affinity receptor for either CGRP or adrenomedullin. The RAMPs are a family of three proteins (Figure 4), each with an extracellular amino terminus of approximately 100 amino acids, a single transmembrane section, and a very short carboxy terminus of approximately 10 amino acids.

### CRLR/RAMP1; A CGRP$_1$ Receptor

CRLR and RAMP1 associate intracellularly and this facilitates their transport to the cell surface. There, the heterodimer functions as a CGRP$_1$ receptor, with a high affinity for CGRP, CGRP$_{8-37}$, and BIBN4096BS. The complex binds adrenomedullin with an approximately 10-fold lower affinity than CGRP. Little is known about the mechanism of ligand binding and receptor activation. CGRP can cross-link to both CRLR and RAMP1, showing that both components create the peptide-binding site. However, it is not clear whether CGRP has specific contacts with both proteins or whether RAMP1 indirectly contributes to the ligand-binding site by modifying the structure of CRLR. Based on studies with other members of the B-family of G protein coupled receptors, it is likely that CGRP makes contacts with both the extracellular domain of CRLR/RAMP1 and the membrane–extracellular loop interfaces of CRLR. The selectivity of BIBN4096BS for primate over rodent CGRP receptors is due to a single amino acid at position 74 in RAMP1; this is tryptophan in humans and lysine in rats.

### CRLR/RAMP2 and CRLR/RAMP3 are Adrenomedullin Receptors

The heterodimers formed by RAMP2 and RAMP3 with CRLR produce adrenomedullin receptors. Their formation is very similar to that of the CRLR/RAMP1 complex; in the absence of CRLR, they are not expressed at the cell surface. It was initially thought that RAMP2 might work by modulating the glycosylation state of CRLR. However, it is now thought that adrenomedullin and CGRP both bind to the fully glycosylated form of CRLR. As with the CRLR/RAMP1 complex, there is little detailed information on mechanisms of adrenomedullin binding to either the CRLR/RAMP2 or the CRLR/RAMP3 complexes. Both complexes exhibit higher affinity for adrenomedullin than CGRP. It has been proposed that the CRLR/RAMP2 complex should be designated as the AM$_1$ receptor and the CRLR/RAMP3 complex as the AM$_2$ receptor. The CRLR/RAMP2 complex has high affinities for adrenomedullin and adrenomedullin$_{22-52}$ but low affinity for CGRP. The CRLR/RAMP3 complex has not been extensively characterized but has high affinity for adrenomedullin and an intermediate affinity for CGRP, leading to the suggestion that it may function as a mixed adrenomedullin/CGRP receptor.

### Cellular Signaling and RCP

The CRLR/RAMP complexes all couple to stimulation of adenylate cyclase production via the G protein known as $G_s$. However, as is the case with many related GPCRs,

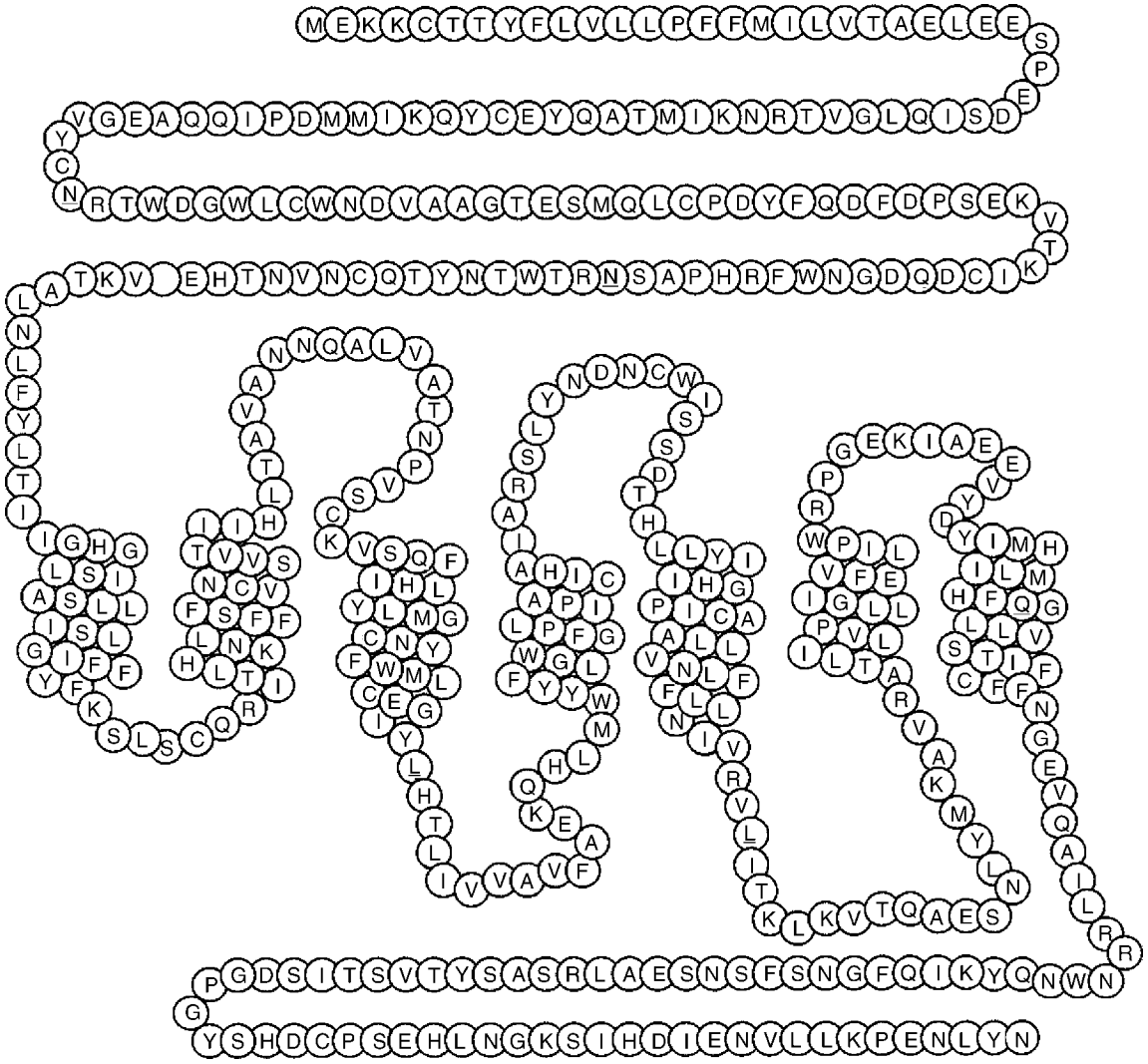


**FIGURE 3** The structure of human CRLR, showing the postulated seven-transmembrane helices. The residues that are likely glycosylation sites on the amino terminus are underlined.

```
RAMP3   -------------METGALRRPQLLPLLLLLCG---------------GCPRAGGCNETG
RAMP2   MASLRVERAGGPRLPRTRVGRPAAVRLLLLLCAVLNPHEALAQPLPTTGTPGSEGGTVKN
RMAP1   -------------MARALCRLPRRGLWLLLAHH----------------LFMTTACQEAN
                                 *     ***

RAMP3   MLERL-PLCGKAFADMMGKVDVWKWCNLSEFIVYYESFTNCTEMEANVVGCYWPNPLAQG
RAMP2   YETAV-QFCWNHYKDQMDPIEK-DWCDWAMISRPYSTLRDCLEHFAELFDLGFPNPLAER
RAMP1   YGALLRELCLTQFQVDMEAVGETLWCDWGRTIRSYRELADCTWHMAEKLGCFWPNAEVDR
                  *       *       **        *     *    *       **

RAMP3   FITGIHRQFFSNCTVDRVHLEDPPDEVLIPLIVIPVVLTVAMAGLVVWRSKRTDTLL
RAMP2   IIFETHQIHFANCSLVQPTFSDPPEDVLLAMIIAPICLIPFLITLVVWRSKDSEAQA
RAMP1   FFLAVHGRYFRSCPISGRAVRDPPGSILYPFIVVPITVTLLVTALVVWQSKRTEGIV
             *   *   *        ***   *   *  *           **** **
                                      TM domain
```

**FIGURE 4** Alignment of human RAMPs 1, 2, and 3. * Single, fully conserved residue. Underlined italic residues at the start of the sequence represent signal peptides. Underlined boldface residues are potential glycosylation sites.

coupling to $G_i/G_o$ and the $G_q$ family of G proteins (leading to changes in ion channel activity and elevation of intracellular calcium) has also been reported.

A novel protein called receptor component protein (RCP) is reportedly essential for coupling the CRLR/RAMP complexes to stimulation of $G_s$. Immunoprecipitation studies suggest that RCP is physically associated with the CRLR/RAMP heterodimer. It has been reported that inhibiting RCP expression disrupts signaling mediated via CRLR/RAMP complexes. RCP is very widely distributed in cell lines but a broader role has not yet been demonstrated.

### Other Receptors that Respond to CGRP and Adrenomedullin

There is pharmacological evidence for heterogeneity among endogenous receptors for both CGRP and adrenomedullin. However, the molecular basis for this remains unclear. Adrenomedullin receptor heterogeneity may be explained at least in part by CRLR/RAMP2 and CRLR/RAMP3 dimers. As the CRLR/RAMP3 complex shows appreciable affinity for CGRP, it may also contribute to the appearance of $CGRP_2$-like pharmacology. RAMPs can also dimerize with calcitonin receptors. These complexes are usually considered to be amylin receptors; however, the RAMP1-containing dimer shows an appreciable affinity for CGRP. In addition, the presence of completely novel receptors for CGRP and adrenomedullin cannot be excluded.

## The Distribution and Physiology of CGRP and Adrenomedullin Receptors

### The Distribution of Binding Sites for CGRP and Adrenomedullin

CGRP-binding sites are present in both the central nervous system (CNS) and peripheral tissues. In some of the earlier literature, there was confusion due to cross-labeling of what would now be considered amylin receptors. Specific CGRP receptors show the potency order: CGRP > adrenomedullin > amylin. In the rat CNS, the highest densities of CGRP binding are in the nucleus accumbens, caudate putamen, amygdaloid body, pontine nuclei, cerebellum, spinal cord, and inferior olive. In the periphery, CGRP receptors are particularly associated with the cardiovascular system, on blood vessels and in the atria. There are high densities of these receptors in the spleen. However, receptors are also found on nonvascular cells, for example, in the vas deferens and secretory cells in the gastrointestinal tract. In tissues, adrenomedullin receptors show a high level of specificity for adrenomedullin over CGRP or other peptides. Binding in the CNS is highest in the spinal cord, but moderate to low levels are seen in many other brain regions. In the periphery, it is closely associated with the cardiovascular system, with very high levels in the heart and lungs.

### Distribution of RAMPs and CRLR

Almost all information on the distribution of CRLR and RAMPs comes from mRNA measurements. Generally, there is fair agreement between the distribution of RAMPs and CRLR and binding sites for CGRP and adrenomedullin. Often RAMP2 appears to be more abundant than RAMP1, with RAMP3 being expressed at the lowest levels. However, there are many exceptions; RAMP1 is the most abundant transcript in the brain and the pancreas and RAMP3 predominates in liver. There are some anomalies; the nucleus accumbens has strong CGRP binding and RAMP1 expression but little CRLR; by contrast, the frontal cortex has very little binding for CGRP, adrenomedullin, or amylin, but has high levels of RAMP expression. It is unclear whether this reflects problems of inferring protein expression from mRNA abundance or whether it indicates additional complexity in the field. There is some preliminary evidence that RAMPs can associate with other receptors in addition to those for calcitonin and CRLR.

### Changes in Expression of CGRP and Adrenomedullin Receptors

There is good evidence for changes in CGRP and adrenomedullin receptors during various (patho)physiological processes. For example, adjuvant-induced arthritis in rats leads to a decrease in CGRP binding in the spinal cord. Changes in CRLR and RAMP expression have also been noted in disease. In a mouse model of sepsis, CRLR mRNA and RAMP2 mRNA show large decreases but RAMP3 expression increases in the lung. In a rat model of ischemic heart failure, RAMP2 expression is increased in the ventricles.

### The Pathophysiology of CGRP and Adrenomedullin Receptors

Changes in receptor expression suggest that both CGRP and adrenomedullin receptors are of importance during normal physiological processes and also in disease. Although receptor knockout models have not yet been described, mice in which the gene for adrenomedullin has been inactivated die *in utero*, showing gross defects in their cardiovascular systems. Heterozygote knockout mice, in which adrenomedullin

production has been reduced, show an elevated blood pressure. Thus, adrenomedullin appears to be vital for normal operation of the cardiovascular system. Similar studies on αCGRP-deficient mice have produced less dramatic effects, although some workers have noted an increased blood pressure, decreased pain sensitivity, and reduced hypersensitivity to antigens in airways. The data are consistent with roles for CGRP in the cardiovascular system and also as an inflammatory mediator, involved in pain perception and the activation of cells of the immune system. Adrenomedullin and CGRP receptors are potentially important therapeutic targets in cardiovascular and inflammatory diseases.

## See Also the Following Articles

Calcitonin Receptor • G Protein-Coupled Receptor Kinases and Arrestins

## Glossary

**agonist** A drug that activates a receptor.

**antagonist** A drug that blocks the action of an agonist at a receptor.

**glycosylation** The attachment of sugars to a protein.

**$pA_2$** The negative logarithm of the antagonist concentration that causes a twofold shift in the agonist dose–response curve. It is a measure of antagonist affinity.

## Further Reading

Brain, S. D., Poyner, D. R., and Hill, R. G. (2002). Calcitonin gene-related peptide (CGRP) receptors: A headache to study, but will antagonists prove therapeutic in migraine? *Trends Pharmacol. Sci.* **23**, 51–53.

Hinson, J. P., Kapas, S., and Smith, D. M. (2000). Adrenomedullin, a multifunctional regulatory peptide. *Endocr. Rev.* **21**, 138–167.

Juaneda, C., Dumont, Y., and Quirion, R. (2000). The molecular pharmacology of CGRP and related peptide receptor subtypes. *Trends Pharmacol. Sci.* **21**, 432–438.

Poyner, D. R., Marshall, I., and Brain, S. D. (2000). *The CGRP Family; CGRP, Amylin and Adrenomedullin*. Eurekah.com, Landes Biosciences, TX.

Poyner, D. R., Sexton, P. M., Marshall, I., Smith, D. M., Quirion, R., Born, W., Muff, R., Fischer, J. A., and Foord, S. M. (2002). International Union of Pharmacology XXXII. The mammalian CGRP, adrenomedullin, amylin and calcitonin receptors. *Pharmacol. Rev.* **54**, 233–246.

Sexton, P. M., Albiston, A., Morfis, M., and Tilakaratne, N. (2001). Receptor activity modifying proteins. *Cell Signal.* **13**, 73–83.

## Biography

David Poyner is a Senior Lecturer in Pharmacology at Aston University. Debbie Hay and Alex Conner are postdoctoral fellows at the same institute. The Aston group has been researching the pharmacology of CGRP and adrenomedullin for over 10 years and in that time has produced over 40 publications in these fields.

# Calcitonin Receptor

**Samia I. Girgis, Niloufar Moradi-Bidhendi, Lucia Mancini and Iain MacIntyre**
*Imperial College and William Harvey Research Institute, London, UK*

**The calcitonin receptor (CTR) is a cell-surface receptor on which the peptide hormone calcitonin binds with high affinity to exert its bio-effects. Calcitonin (CT) has a wide range of effects, the most obvious of which is its effect on bone. CT is a powerful inhibitor of bone resorption. It directly inhibits the activity of the osteoclast (special bone cell responsible for bone resorption). CT receptors are widely distributed and CT has a much broader range of biological activities, including the effects on the central nervous system (CNS), gastrointestinal tract, and vascular and immune systems. Peptides that are structurally similar to CT, known as the CT peptide family bind with lower affinities to the CT receptors. The CTR has seven transmembrane-domains, and belongs to the G protein-coupled family of receptors (GPCRs).**

## Calcitonin Peptide Family

The calcitonin family of peptides comprises five known members, which are structurally similar (Figure 1). They have some overlapping biological effects due to cross-reactivity at each other's receptors. The CT peptide family includes calcitonin, two calcitonin gene-related peptides, amylin, and adrenomedullin, which are discussed next.

### Calcitonin

Calcitonin (CT) is a peptide hormone secreted mainly by special type of cells within the thyroid gland in mammals, called C cells. CT was first identified as a calcium-lowering factor. This calcium-lowering effect of CT is mainly due to inhibition of osteoclast activity, and to a lesser extent to increasing calcium excretion by the kidney. As mentioned previously, CT also has a wide range of biological activities, including its analgesic effect, suppression of gastric acid secretion, and appetite.

### Two Calcitonin Gene-Related Peptides (CGRP 1 and CGRP 2)

These are neuropeptides widely distributed throughout the CNS and in peripheral nerves associated with the cardiovascular system. The tissue distribution suggests a role in the transmission of sensory impulses and in the regulation of vascular tone. Both are very potent vasodilators.

### Amylin

This is found in secretory granules in $\beta$-cells of the pancreas (cells that secrete insulin) and is co-secreted with insulin in response to a high blood glucose, e.g., after a meal. Amylin is a potent inhibitor of gastric emptying and food intake. It also opposes the metabolic actions of insulin in skeletal muscle.

### Adrenomedullin (ADM)

This is produced predominantly by the vascular endothelium. It is a potent vasodilator.

## The CT Receptor (CTR)

### Distribution

Calcitonin receptors (CTRs) are widely distributed. They are particularly numerous in osteoclasts, where there are ~1.3 million receptors per cell. They are found in lower amounts in the CNS, in areas involved in the control of appetite, pain perception, and lactation, e.g., hypothalamus and pituitary. CTRs are also found in peripheral tissues, e.g., in cells of the distal nephron of the kidney, testes, placenta, lung, prostate, and lymphocytes. Receptors for CT have been identified in some human cancer cells, including those of lung, breast, bone, and prostate.

### Protein Structure

The CTR was initially cloned from a pig kidney cell line. Analysis of the predicted 482 amino acid sequence demonstrated seven hydrophobic regions that could generate transmembrane (TM)-spanning domains. The CTR is a member of a subset of the G protein-coupled receptors (GPCRs) family termed GPCR$_B$. Members of this family typically recognize regulatory peptides, including parathyroid hormone (PTH), glucagon, and secretin.

 © 2004, Elsevier Inc. All Rights Reserved. 

| | |
|---|---|
| Human CGRP$\alpha$ | A **C** D T A T **C** V T H R L A G L L S R S G G V V K N N F V P T N V G S K A F-**amide** |
| Human CGRP$\beta$ | A **C** N T A T **C** V T H R L A G L L S R S G G M V K S N F V P T N V G S K A F- **amide** |
| Human AMY | K **C** N T A T **C** A T Q R L A N F L V H S S N N F G A I L S S T N V G S N T Y **-amide** |
| Human ADM | Y R Q S M N N F Q G L R S F G **C** R F G T **C** T V Q K L A H Q I Y Q F T D K D K D N V A P R S K I S P Q G Y **-amide** |
| Human CT | **C** G N L S T **C** M L G T Y T Q D F N K F H T F P Q T A I G V G A P **-amide** |

**FIGURE 1** The CT peptide family. The peptides share a six/seven amino acid ring structure, formed by a disulphide bridge at or close to the N terminus. The peptides also have C-terminal amides, which are essential for full biological activity. (This material is from Calcitonin Gene-related Peptide (CGRP), Girgis, S. I., and MacIntyre, I. (2002). In *Encyclopedia of Molecular Medicine* (T.E. Creighton ed.), Vol. 5, John Wiley & Sons Inc.

This family of receptors is characterized by seven TM segments connected by three extracellular and three intracellular loops (Figure 2). They all have an extracellular amino (N)-terminal sequence and an intracellular carboxy(C)-terminal sequence. The extended extracellular N-terminal region contains multiple potential glycosylation sites and conserved cysteine residues. Glycosylation is important for recognition and high-affinity binding of CT. While the TM domain sequences are conserved (40–60% identical), the N-terminal domains are generally less than 25% identical. The N-terminal domain fulfills the role for ligand binding and receptor specificity.

## Multiple Forms

The human CTR gene is localized to chromosome 7. The CTR gene has a complex structural organization with several CTR protein isoforms derived from alternative splicing of transcripts from a single gene. These isoforms are functionally distinct in terms of ligand-binding specificity and signal transduction pathway utilization, and tissue distribution. In humans at least five splice variants have been described whereas two splice variants have been identified in rodents (C1a and C1b), which differ by the presence or absence of an additional 37 amino acids in the second extracellular domain. The C1a is more widely distributed and is the predominant isoform found in osteoclasts. C1b isoform is primarily localized in the central nervous system.

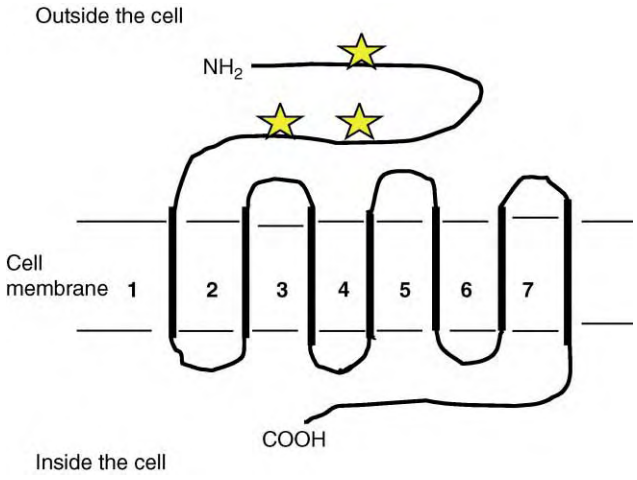



**FIGURE 2** Schematic representation of the porcine CTR. Seven transmembrane regions span the cell membrane, and are connected by three extracellular and three intracellular loops. The amino-terminal extracellular loop contains three glycosylation sites (indicated by stars) and is involved with CT recognition and binding. The intracellular domain is involved with G protein coupling.

## Signaling

The CTR is coupled to multiple signal pathways through interaction with different members of the heterotrimeric G protein family. These are a family of plasma membrane regulatory proteins that many activated receptors interact with and alter. The main signal pathways are the adenylate cyclase/cAMP/protein kinase A and the phosphoinositide-dependent phospholipase C (PLC) pathway, which are discussed next.

### The Adenylate Cyclase/cAMP/Protein Kinase A

The binding of CT to its receptor induces conformational change in the receptor that allows it to bind to an adjacent membrane G-protein, known as $G_s$ (the subscript s denotes stimulation). The binding causes the $G_s$ protein to activate the membrane enzyme called adenylate cyclase. The activated adenylate cyclase, whose catalytic site is on the cytosolic site of the plasma membrane, then catalyses the conversion of cytosolic ATP to cyclic 3′,5′ adenosine monophosphate (cAMP). Cyclic AMP can diffuse throughout the cell to trigger the sequence of events leading to the cell's ultimate response to CT.

### The Phosphoinositide-Dependent Phospholipase C (PLC) Pathway

The PLC pathway results in both an increase in intracellular $Ca^{2+}$ and protein kinase C (PKC) activation via $G_q$ protein. Both cAMP and intracellular $Ca^{2+}$ are important second messengers for mediating the actions of CT on the osteoclast.

While adenylate cyclase, PLC and PLD are established effectors for CTR, recently CTR mediated activation of the mitogen-activated protein kinase (MAPK) pathway has been described. MAPKs are a group of serine/threonine protein kinases that are activated by several distinct classes of cell surface receptors including G-protein coupled receptors and tyrosine kinases.

## Regulation

Regulation of the level or affinity of cell-surface receptors is a key component in the response to either endogenous or administered agents. The CTR is subject to both homologous (CT-induced) and heterologous regulation. Although CT effectively inhibits osteoclast-mediated bone resorption after acute administration, continuous exposure to CT results in a loss of responsiveness due to receptor down-regulation. Continuous treatment of osteoclast-like cells with CT results in the decrease in steady state levels of CTR mRNA and down-regulation of CTR binding.

It is also known that glucocorticoids stimulate CTR expression.

## CTR-Like Receptor (CTRLR)

This receptor has a significant sequence homology (55%) with the CTR and share similarities in general structure and length. The CTRLR is expressed in a variety of tissues, being most prevalent in the brain, and in high densities in the pulmonary and cardiovascular system (especially in blood vessels). It is also expressed

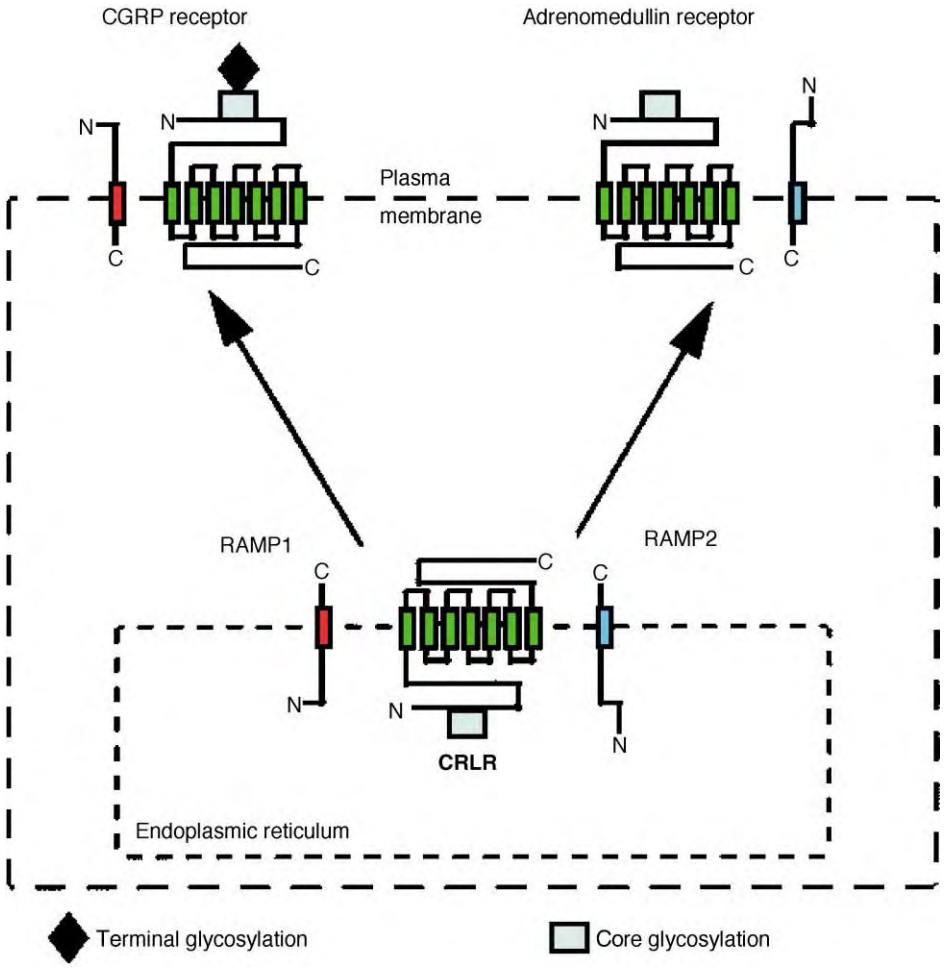



**FIGURE 3** The role of RAMPs 1 and 2 and CRLR in generating CGRP or ADM receptors. (Reproduced from McLatchie L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G., and Foord, S.M. (1998). RAMPs regulate the transport and ligand specificity of the CRLR. *Nature* (*Lond.*) **393**, 333–339.)

in the adrenal and pituitary glands, exocrine pancreas, kidney, and bone. Surprisingly, it does not bind with high affinity to any of the members of the CT peptide family. It requires other cell membrane proteins called receptor activity-modifying proteins (RAMPs) to modify its affinity for the different peptide of the CT peptide family (Figure 3).

RAMPs are single TM proteins. There are three members: RAMP1, RAMP2, and RAMP3. RAMP I acts to modify the glycosylation of CTRLR, enhance the transport of the receptor protein to the cell surface and potentially contribute to the cell surface phenotype of the receptor. RAMP I-transported CTRLR is a CGRP receptor. RAMP 2 and 3 also enhance the transport of CTRLR to the cell surface, but do not alter the pattern of glycosylation of the receptor. RAMP 2 or 3-CTRLR is an ADM receptor (Figure 3). They also act to modify the affinity of the CTR for the different peptide ligands of the CT peptide family.

## See Also the Following Articles

Calcitonin Gene-Related Peptide and Adrenomedullin Receptors • Phospholipase C

## Glossary

**affinity** The strength with which a chemical messenger binds to its receptor.

**calcitonin (CT)** A small peptide hormone and a potent inhibitor of bone resorption.

**down-regulation** A decrease in receptor number resulting from prolonged exposure to high concentration of the message.

**G proteins** A family of plasma membrane regulatory proteins that many activated receptors interact with and alter.

**osteoclast** A large multinucleated bone cell that can destroy bone.

**phospholipase C** Enzyme that catalyzes the breakdown of a plasma membrane phospholipid known as phosphatidyl-inositol bisphosphate ($PIP_2$) to inositol triphosphate ($IP_3$) and diacylglycerol (DAG).

**protein kinases** Enzymes that phosphorylate other proteins by transferring to them a phosphate group from ATP. Introduction of a phosphate group alters the activity of the protein, often itself an enzyme.

**receptor-activity-modifying proteins (RAMPs)** Single transmembrane-domain proteins that alter the phenotype of the calcitonin receptor-like receptor.

**second messengers** Substances that serve as the relay from the plasma membrane to the biochemical machinery inside the cell.

## Further Reading

Christopouos, G., Perry, K. J., Morfis, M., Tilakaratne, N., Gao, Y., Fraser, N. J., Main, M. J., Foord, S. M., and Sexton, P. M. (1999). Multiple Amylin receptors arise from receptor activity – modifying protein interaction with the Calcitonin receptor gene product. *Am. Soc. Pharm. Exp. Ther.* **56**, 235–242.

Galson, D. L., and Goldring, S. R. (2002). Structure and molecular biology of the calcitonin receptor. In *Principle of Bone Biology*, (J. P. Bilezikian, L. G. Raisz, and G. A. Rodan, eds.) 2nd edition, Vol 1, pp. 603–617. Academic Press, California.

McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M. G., and Foord, S. M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin receptor-like receptor. *Nature (Lond.)* **393**, 333–339.

Pondel, M. (2000). Calcitonin and calcitonin receptors: Bone and beyond. *Int. J. Exp. Pathol.* **81**, 405–422.

Poyner, D. R., Sexton, P. M., Marshall, I., Smith, D. M., Quirion, R., Born, W., Muff, R., Fischer, J. A., and Foord, S. M. (2002). International union of pharmacology: XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol. Rev.* **54**, 233–246.

Sexton, P. M., Findlay, D. M., and Martin, T. J. (1999). Calcitonin. *Curr. Med. Chem.* **6**, 1067–1093.

## Biography

Samia I. Girgis is a Senior Lecturer in the Department of Metabolic Medicine at Imperial College London, Hammersmith Campus, and an Honorary Consultant Chemical Pathologist at the Hammersmith Hospitals NHS Trust. For many years, her research interest has been concerned with the calcitonin gene peptide family. This included the isolation, characterization and measurement of these peptides. Her recent research interest has focused on biochemical markers of bone metabolism, their potential role in diagnosis and monitoring of patients with metabolic bone diseases.

Niloufar Moradi-Bidhendi is a Post-doctoral Research Assistant in the William Harvey Research Insitute. In 2001 she was awarded a Ph.D. on "The Interrelation of Nitric Oxide and 17ß-Oestradiol in Bone Cells." Her research interests have focused mainly on bone metabolism although her more recent studies have included work on the role of nitric oxide in wound healing.

Lucia Mancini is a Post-doctoral Research Assistant in the William Harvey Research Institute. In 2000 she was awarded a Ph.D. on "Nitric Oxide Regulation of Bone Metabolism." She is currently working in the Centre of Biochemical Pharmacology on a project examining the effect of calcitonin on the regulation of the RANK/OPG system.

Iain MacIntyre is Director of Research in the William Harvey Research Institute. After qualifying in medicine in Glasgow he trained in biochemistry as a demonstrator in Professor Hans Krebs' laboratory in Sheffield and thereafter he became Director of the Endocrine Unit at Hammersmith. His research interests have always centered on calcium and after the co-discovery of calcitonin and the definition of the thyroid as its gland of origin, he contributed many research papers on the chemistry, physiology, and clinical relevance of calcitonin. His more recent interests have focused on nitric oxide and its interaction with estrogen.

# Calcium Buffering Proteins: Calbindin

**Willi Hunziker**
*Frimorfo SA, Fribourg, Switzerland*

**Igor Bendik**
*DSM Nutritional Products, Kaiseraugst, Switzerland*

**The calbindins, calbindin D-9k and calbindin D-28k, belong to a large family of intracellular $Ca^{2+}$-binding proteins of more than 300 members classified in 45 distinct EF-hand subfamilies. Their names relate to their early discovery based on calcium binding and vitamin-D responsive expression. The calbindins are mainly localized in the cytosolic compartment of the cells and exert their action after binding ionized calcium ($Ca^{2+}$). Calcium ions are important biological regulators that trigger a wide array of processes. Therefore, the handling of $Ca^{2+}$, i.e., the $Ca^{2+}$-absorption, -transport, -buffering, -storage, -distribution, -signaling, and -sensing are fundamental for higher life. Functionally, the large family of EF-hand calcium-binding proteins can be classified into (1) calcium-sensing proteins, which propagate a cellular signal, (2) calcium transport proteins which are involved in the cellular calcium transport, and (3) calcium buffer proteins, that are believed to buffer the intracellular free calcium, to prevent it from damaging the cells. The calbindins are believed to be involved in some of these essential processes but their exact physiological function remains still to be determined.**

## Calbindin D-9k

### History

Originally, calbindin D-9k and calbindin D-28k have been identified during the study of vitamin-D-dependent calcium absorption. In 1966 Wassermann and Taylor reported an intracellular vitamin D-dependent calcium-binding protein in the chicken intestine. One year later, Kallfelz and colleagues observed that this 28 kDa protein was not present in the mammalian intestine, but instead they identified a vitamin D-regulated 9 kDa intestinal calcium-binding protein that was later on called calbindin D-9k. Calbindin D-9k seems to be an evolutionary younger protein, since it does not seem to exist in birds. In mammals calbindins D-9k and D-28k have a dissimilar tissue distribution. Calbindin D-9k is highly abundant in the small intestine, whereas calbindin D-28k is absent, but it is predominant in brain and kidney. Vitamin D-induced calbindin expression is only observed in organs involved in $Ca^{2+}$ homeostasis like intestine and kidney, in the brain calbindin D28 is present independent of vitamin D.

After its identification in 1967 calbindin D-9k was initially called calcium-binding protein 9 kDa, CaBP9k, or intestinal calcium binding protein (ICBP). Five years later the first polyclonal antibodies were generated that allowed localization and tissue distribution studies of calbindin D-9k. In 1983 the rat cDNA clone of calbindin D-9k was isolated, whereas the human counterpart was identified later in 1993. The calbindin D-9k protein is characteristic for mammals but seems to be absent in birds. It consists of two calcium-binding domains and is more closely related to S100 proteins than to calbindin D-28k. Members of the S100 protein family, a subfamily of intracellular $Ca^{2+}$-binding protein family, are typically small, acidic proteins containing two $Ca^{2+}$-binding sites. Each member of the S100 protein family has a unique spatial and temporal expression pattern. The structural relatedness seems not to coincide with its location and function.

### Distribution and Localization

Calbindin D-9k is only found in mammals where it is highly abundant in the small intestine, and at lower levels also in placenta, uterus, kidney, yolk sac, fallopian tube, lung, cartilage, bone, and teeth. In the intestine the highest calbindin D-9k concentration is found in the cytoplasma of duodenal villus enterocytes. The expression levels in juvenile tissues are substantially higher than in adult ones. Antisera raised against intestinal calbindin D-9k from one species generally do not crossreact with calbindin D-9k from other species. This is likely due to the lower amino acid sequence conservation of calbindin D9 in evolution as compared to that of calbindin D28.

 © 2004, Elsevier Inc. All Rights Reserved. 

### Gene and Sequence

The human calbindin D-9k is a heatstable and acidic ($pI = 4.52$) protein consisting of 79 amino acid (9016 Da). The human protein has an amino acid sequence identity of 78% and 74% to that of the rat and that of the mouse, respectively. The first cDNA clone was identified in 1983 by differential *in situ* ybridization in the rat system. The structural organization of the calbindin D-9k gene was determined five years later. The chromosomal location was assigned to Xp22.2 and the gene name was defined to CALB3. The CALB3 gene is a small gene of 5.5 kb comprising three exons, the coding region is contained in the exons two and three, which code for the two $Ca^{2+}$-binding sites, respectively.

### Calcium Binding

The X-ray structure of the $Ca^{2+}$-binding sites of calbindin D-9k was first resolved for the bovine protein. Calbindin D9-k contains two helix-loop-helix structures of 29 and 31 amino acids called EF-hands, each binding one $Ca^{2+}$ ion. These EF-hand structures are similar to those found in the S100 proteins and thus, calbindin 9k is considered to be a member of the S-100 protein family. Characteristic for the S100 protein family members is the atypical second EF-hand with a 14 amino-acid loop, which is different to the prototype EF-hand having a loop of 12 amino acids. Calbindin D-9k reportedly binds $Ca^{2+}$ without a significant change in conformation. The equilibrium binding constant for calbindin D-9k is in the high nanomolar range.

### Regulation

Calbindin D-9k is believed to play an important role in the $Ca^{2+}$ absorption by transporting and/or buffering cytoplastic $Ca^{2+}$. Vitamin D plays a pivotal role in this regulation. The hormonally active metabolite of vitamin D, $1,25(OH)_2D_3$ (calcitriol), rapidly induces calbindin D-9k synthesis in the mammalian intestine. Calcitriol regulates the expression of calbindin D-9k not only at the transcriptional but also at the translational level. For transcriptional regulation calcitriol binds the nuclear vitamin D receptor (VDR), which is a DNA-binding protein that belongs to steroid receptor superfamily. The liganded VDR recognizes a specific VDR-response element in the promoter of the calbindin D-9k gene and activates its expression. A similar regulation of expression is believed to take place in the kidney, placenta, bone, and teeth. In the uterus calbindin D-9k is regulated by estradiol but not by vitamin D. Expression of calbindin D-9k is undetectable in the uterus in the absence of estradiol. If estradiol is present, it binds to the estrogen receptor and activates expression of the calbindin D-9k gene in a mechanism similar to that described above for vitamin D. In the lung calbindin D-9k is expressed independent of vitamin D and estradiol.

### Summary and Outlook

Calbindin D-9k is a calcium-binding protein with two calcium-binding domains which belongs to the S-100 EF-hand protein family. It has a characteristic tissue distribution in mammals and does not seem to exist in birds. Its expression is regulated by calcitriol, the active metabolite of vitamin D, mainly in intestine and kidney, whereas it is regulated by estrogens in the uterus. It is thought to be involved in the transport of calcium. To our knowledge, gene knockout mice have thus far not yet been generated and no human genetic disease based on a calbindin D-9k defect has been described, both of which would allow to gain further insight on the function of this protein.

## Calbindin D-28k

### History

In the chicken intestinal mucosa Wassermann and co-workers identified a vitamin D-inducible 28 kDa protein that makes up from 1% to 3% of all cytosolic proteins. This protein was referred to vitamin D-dependent calcium-binding protein or CaBP. Heat treatment to 80°C did not result in significant alterations of its immunological, electrophoretical, or calcium-binding properties. As a matter of fact, a heating step was used in some of the early calbindin D-28k purification protocols. It took 20 years from its original discovery until the calbindin D-28k cDNA was cloned and sequenced. The amino acid sequence implied a 6 EF-domain structure of which two domains have lost their $Ca^{2+}$-binding function. This finding confirmed previous observations that calbindin D-28k binds 3–4 moles of $Ca^{2+}$. Calbindin D-28k shares only minimal sequence homology with calbindin D-9k and the S-100 family, but is rather closely related to calretinin, another 6 EF-hand domain calcium-binding protein expressed mainly in neuronal tissues. Calbindin D-28k is highly conserved during evolution suggestive of an important physiological function that allows only little sequence divergence. The exact physiological function, however, remains still unclear.

### Distribution and Localization

Calbindin D-28k is expressed in brain, kidney, bone, pancreas, and less in some other tissues (pituitary gland, salivary gland, adrenal gland, stomach, thymus, bladder, ovary, heart, liver). The expression level between tissues varies from 0.1% to 1.5% of the total soluble protein. Calbindin D-28k is expressed in specific

regions throughout the central nervous system. The highest calbindin D-28k levels are present in the cerebellar Purkinje cells where it is located in the cell bodies, axons, and dendrites, but not or to a much lesser extent in the adjacent granular cell layer. Furthermore, calbindin D-28k is localized in specific cells of the sensory pathways in mammalian cochlear and vestibular hair cells in the inner ear, as well as in the basilar papilla of the chick cochlea. Calbindin D-28k concentrations of up to 2 mM were reported to be present in the auditory neurons. In the kidney, calbindin D-28k is exclusively localized in the tubular regions of the distal nephron (distal convoluted tubule, the connecting tubule, and the cortical collecting tubule), where reabsorption of calcium is known to occur. Calbindin D-28k is also found to be expressed in the insulin-producing B cells of the avian and mammalian pancreas.

Calbindin D-28k is expressed in the avian but not in the mammalian intestine, where it is found by immunocytochemical techniques to be present in the intestinal absorptive cells whereas globlet cells are negative. The subcellular distribution of calbindin D-28k was determined in chick duodenum by electron microscopy using the protein A-gold technique. Calbindin D-28k was found in the cytosol and the nuclear euchromatin of the intestinal absorptive cells, but not in the mitochondria, the lysosomes, or the cysternal spaces of the rough endoplasmic reticulum nor in the Golgi apparatus.

An antiserum raised to chick intestinal calbindin D28 was found to also crossreact with calbindin D-28k from other species. The antiserum revealed the presence of calbindin D-28k in intestinal homogenates of duck, robin, and Japanese quail and with a protein in the duodenum of reptiles but not fishes and amphibia. Similarly, calbindin D-28k was also identified in the kidney and brain of amphibia, reptiles, birds, and mammals and also in the nervous system of fish and several mollusks. Since calbindin D-28k is found in fish brain but not in fish intestine and kidney, one might speculate that it was originally a neuronal protein.

## Gene and Sequence

The rigid structure of calbindin D-28k is acidic ($pI = 4.54$) and contains 261 amino acids (30 025 Da). The chicken cDNA sequence was the first to be cloned (1986). The deduced amino acid sequence of this calbindin D-28k cDNA revealed surprisingly the presence of six EF-hand domains. Of these predicted six EF-hand domains only four were considered to be functional due to mutations within the conserved calcium coordination sites in the loop regions of domains 2 and 6. A protein sequence comparison of calbindin D-28k showed a high degree of conservation over all species. The human calbindin D-28k gene was localized to the human chromosome 8q21.3–q22.1 and the gene name was annotated to CALB1. CALB1 spans 24 kb interspersed by 10 introns and 11 exons.

## Calcium Binding

EF-hand proteins play a crucial role to guide and sustain the calcium signal. The designation "EF hand" is derived from the structural orientation of the two $\alpha$-helices (E and F) that form together with the calcium-binding loop of 12 amino acids the highly conserved metal-binding consensus sequence. The loop contains five oxygen-containing amino acids at defined positions that are important for coordinating the calcium ion. Da Silva and Reinach proposed to classify the EF-hand members into proteins with regulatory roles called $Ca^{2+}$ sensor proteins and those involved in $Ca^{2+}$ buffer and transport were entitled $Ca^{2+}$ buffer proteins. The $Ca^{2+}$-binding affinities of EF-hand proteins have a wide equilibrium constant range ($K_d = 10^{-4}$–$10^{-9}$ M). This large binding range is coded in the amino acid sequence, in particular within the 12-residues consensus loop that directly coordinates the $Ca^{2+}$ ion. The four EF-hands domains of calbindin D-28k bind calcium with high affinity. The binding is $Ca^{2+}$-specific with marginal $Ca^{2+}/Mg^{2+}$ antagonism. Therefore, under resting intracellular calcium concentrations ($<100$ nM) the calcium-binding sites of calbindin D-28k are largely empty and are thus immediately available for a fast buffering of nerve stimulation evoked $Ca^{2+}$ transients. Two $K_d$ values have been determined calbindin D-28k. The first $K_d(1)$ value ranges from 175 to 237 nM and the second $K_d(2)$ ranges from 411 to 513 nM, respectively. $Ca^{2+}$ binding seemingly causes a conformational change in the molecule, which could trigger an interaction with another protein, thereby propagating a calcium signal.

## Knockout Animals

The evaluation of the physiological role of calbindin D-28k has been facilitated by the recent generation of mouse strains deficient in this protein. Calbindin D-28k-nullmutant (−/−) mice are viable and appeared phenotypically normal in their general development. There was no evidence of any major changes in the histology neither of their nervous system nor of any other organ. However, calbindin D-28k knockout mice showed a distinct motor coordination problem that could not be overcome by continuous learning. These animals lack the calbindin D-28k in the Purkinje cells of the cerebellum, a center for motor coordination. This result points towards a critical physiological role of calbindin D-28k in controlling Purkinje cell-dependent motor behavior.

Due to its presence in Purkinje cells calbindin D-28k was also thought to be associated with other neurological disorders such as ataxia. Patients with spinocerebellar ataxia type 1 (SCA1) show a decreased calbindin D-28k staining in Purkinje cells and an aggregation of ataxin-1 in the nucleus, leading eventually to Purkinje cell death. A similar decrease in calbindin D-28 staining of Purkinje cells was found in transgenic mice overexpressing mutant ataxin-1, an animal model for SCA1. Calbindin D-28k knockout mice on the other hand did not show any Purkinje cell loss, suggesting that the lowered calbindin D-28k levels in SCA1 patients are unlikely the cause but rather the consequence of the Purkinje cell damage due to the high ataxin-1 levels. The fact that the Purkinje cells and other calbindin D-28k positive neurons of wild type mice also survive in the calbindin knockout mice, shows that the lack of calbindin D-28k is not per se detrimental to these cells. Thus, this finding argues against a general calcium-mediated excitotoxicity preventive role of calbindin D-28k. The functional data available thus far suggests that in the cerebellum calbindin D-28k plays a role in motor coordination. To our knowledge, no human genetic disease is known where the root cause is a calbindin D-28k gene defect.

## Regulation

Calbindin D-28k has a unique tissue-specific expression pattern indicating a highly specialized function. Calbindin D-28k is regulated in a vitamin D-dependent fashion in kidney and pancreas, whereas its presence in the specific brain regions is independent of the vitamin D status. There are, however, reports showing a brain region-specific regulation by gluccocorticoids, by sex steroids and by nerve growth factor.

The initial finding that calbindin D-28k is induced by vitamin D in tissues involved in calcium transport, suggested that calbindin D28 might have an important role in vitamin D regulated calcium absorption. The intestine, the kidney, and the bone are target organs for the biologically active form of vitamin D, the 1$\alpha$, 25-dihydroxyvitamin D3, that is known to regulate the calcium demand of the body. The active vitamin D metabolite regulates the expression of calbindin D-28k in these tissues by the steroid hormone mechanism of action as described earlier for calbindin D-9k.

## Summary and Outlook

The fact that antibodies to chicken calbindin D-28k crossreact with calbindin D-28k of evolutionary far-diverged species suggest a high conservation of this protein in evolution. This finding is further evidenced by sequence comparison among the different species. The four intact and the two degenerated EF-hands show similarly a high degree of conservation, suggesting, apart from $Ca^{2+}$-binding, an additional functional conservation pressure. Such an additional function could be triggered by protein–protein interaction in a $Ca^{2+}$-dependent manner. Indeed, there is increasing evidence that similar to calmodulin, also calbindin D-28k could act as calcium sensor, undergoing a conformational change upon $Ca^{2+}$-binding. Such a conformational change could unmask new domains and allow calbindin D-28k to interact with other yet to be identified intracellular effectors thereby propagating the $Ca^{2+}$-signal.

Another intriguing question regarding calbindin D-28k function is the partial substitution during evolution by calbindin D-9k. In mammals calbindin D-9k substitutes for calbindin D-28k in the intestine and in part in the kidney. This raises the intriguing hypothesis that in the evolutionary older species calbindin D28k could have a dual function, one for calcium absorption in the intestine (vitamin D dependent), the other as calcium sensor in neurons (vitamin D-independent). With the evolution of mammals, the calcium absorption function apparently was delegated the "newly invented" calbindin D-9k.

## See Also the Following Articles

Calcium-Binding Proteins: Cytosolic (Annexins, Gelsolins, $C_2$-Domain Proteins) • Calcium Buffering Proteins: ER Luminal Proteins • Calcium Sensing Receptor • Calcium Signaling: Cell Cycle • Calcium Transport in Mitochondria • Vitamin D • Vitamin D Receptor

## Glossary

**EF-hand calcium-binding site** A specific protein sequence constisting of two $\alpha$-helices connected by a sequence of 12–14 amino acids that constitute a high affinity calcium-binding site ($K_d$ in the nM range). Depending on the protein sequence, the binding site is either specific for calcium (e.g., calbindin) or can also accept magnesium (e.g., parvalbumin).

**gene family** A number of closely related genes that arose by a series of gene duplications during evolution. Gene families can have from only very few up to several hundred members. The EF-hand family of calcium-binding proteins has more than 300 members grouped in several subfamilies.

## Further Reading

Bouillon, R., Van Cromphaut, S., and Carmeliet, G. (2003). Intestinal calcium absorption: Molecular vitamin D mediated mechanisms. *J. Cell Biochem.* **88**, 332–339.

Hemmingsen, C. (2000). Regulation of renal calbindin-D28k. *Pharmacol. Toxicol.* **87**(suppl. 3), 5–30.

Schwaller, B., Meyer, M., and Schiffmann, S. (2002). "New" functions for "old" proteins: The role of the calcium-binding proteins

calbindin D-28k, calretinin and parvalbumin, in cerebellar physiologycalbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. *Cerebellum* **1**, 241–258.

## Biography

Willi Hunziker holds a DVM degree from the University of Zürich, Switzerland. He did postdoctoral fellowships at Baylor College of Medicine in Houston, TX and at University California in Riverside. After heading an R&D department at Big Pharma, he is currently CEO of Frimorfo, a Swiss Biotech Service Company (www.frimorfo.com). His research interests are calcium metabolism and regulation of gene expression by steroids, retinoic acid, and vitamin D.

Igor Bendik obtained his Ph.D. from the University of Basel, Switzerland. During his Ph.D. thesis he was interested in calbindin D-28k and the vitamin D receptor. At the Burnham Institute in La Jolla, California, he expanded his knowledge on nuclear receptors. Back in Basel he was a group leader in the medical faculty working on tumor suppressor genes. He is currently employed by DSM, a company active in life sciences, performance materials, and industrial chemicals.

# Calcium Buffering Proteins: ER Luminal Proteins

**Jody Groenendyk and Marek Michalak**
*University of Alberta, Edmonton, Alberta, Canada*

**$Ca^{2+}$ buffering endoplasmic reticulum (ER) proteins are ER resident proteins that bind $Ca^{2+}$ with a high capacity. They play an important role in the maintenance of resting free $Ca^{2+}$ concentration in the lumen of ER and subsequently in the modulation of cellular $Ca^{2+}$ homeostasis. This is critical because $Ca^{2+}$ is a universal intracellular signaling molecule, which controls numerous developmental and cellular pathways. Changes in $Ca^{2+}$ concentration affect processes as diverse as learning and memory, secretion, contraction and relaxation, membrane excitability, cell motility, cytoplasmic and mitochondrial metabolism, protein and lipid synthesis, cell cycle, apoptosis, organellar trafficking, and protein folding and quality control. Therefore, it is of the utmost importance that $Ca^{2+}$ homeostasis is tightly regulated in all cells. This is accomplished by a variety of $Ca^{2+}$ transporting, binding, and buffering proteins, which are found in many cellular compartments.**

## The Endoplasmic Reticulum and $Ca^{2+}$ Homeostasis

The ER is one of the most important intracellular $Ca^{2+}$ buffering and storage organelles. In response to a variety of external stimuli, $Ca^{2+}$ is released from the lumen of the ER into the cytosol, via the inositol-1,4,5-triphosphate receptor ($InsP_3R$) and/or the ryanodine receptor (RyR) $Ca^{2+}$ channels. Some $Ca^{2+}$ also enters the cytosol from the extracellular environment, via $Ca^{2+}$ channels in the plasma membrane. Most of the released $Ca^{2+}$ is taken up back into the lumen of the ER via the sarcoplasmic–endoplasmic-reticulum $Ca^{2+}$-ATPase (SERCA). Some of the $Ca^{2+}$ is also removed from the cytosol by the plasma membrane $Ca^{2+}$-ATPase and the $Na^{+}$–$Ca^{2+}$ exchanger. Within the ER, $Ca^{2+}$ is bound to and buffered by high concentrations of luminal $Ca^{2+}$ binding proteins.

## $Ca^{2+}$ Buffering in the ER Lumen

In total, the lumen of the ER contains 1–3 mM $Ca^{2+}$, a concentration similar to that in the extracellular space. A small portion of this ER lumenal $Ca^{2+}$ is not buffered by $Ca^{2+}$ binding to proteins and is considered free. Resting free $Ca^{2+}$ concentrations in the ER vary from 100 to 400 μM, while those in the cytosol are in the nanomolar range. The actual resting free $Ca^{2+}$ concentration in the ER is determined by the balance between the rates of $Ca^{2+}$ uptake and $Ca^{2+}$ leak and by the extent of buffering by the resident $Ca^{2+}$ binding proteins.

Most $Ca^{2+}$ in the lumen of the ER is bound to the $Ca^{2+}$ binding molecular chaperones that are resident there. These proteins maintain specific concentration gradients of $Ca^{2+}$ in the ER and localize $Ca^{2+}$ to the sites of release. They also directly affect both the $Ca^{2+}$ storage capacity of the ER and the free $Ca^{2+}$ concentration under resting conditions and after cellular stimulation. Table I shows a list of ER proteins that are involved in buffering $Ca^{2+}$ in the ER lumen. There are two major classes of ER $Ca^{2+}$ binding proteins. Class I, the larger of the two, represents proteins which bind $Ca^{2+}$ with relatively high capacity and low affinity. These proteins contain a large proportion of acidic amino acid residues that are involved in the high capacity and low affinity $Ca^{2+}$ binding. Their $Ca^{2+}$ binding capacity varies from as little as 2 moles of $Ca^{2+}$ per mole of protein to as much as 50 moles of $Ca^{2+}$ per mole of protein. The high capacity $Ca^{2+}$ binding sites have a relatively low affinity for $Ca^{2+}$ ($K_d = 1–2$ mM), which parallels the relatively high content of $Ca^{2+}$ in the ER lumen. These Ca binding proteins are responsible for $Ca^{2+}$ storage in the lumen of the ER/sarcoplasmic reticulum (SR), and they effectively determine the capacity of these intracellular organelles for $Ca^{2+}$. In contrast, class II represents $Ca^{2+}$ binding proteins in the lumen of the ER that bind $Ca^{2+}$ with relatively high affinity and low capacity. The function of these high affinity binding sites is not obvious, given the high concentrations of $Ca^{2+}$ in the lumen of the ER. It is thought that they might play a structural role, or that they might be responsible for the regulation/maintenance of specific protein–protein interactions. Several of the class II proteins contain a classic EF-hand

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**$Ca^{2+}$ Binding Proteins of the Endoplasmic Reticulum Lumen**

| Protein name | $Ca^{2+}$ binding (mole/mole of protein) |
|---|---|
| **CLASS I proteins** (high capacity and low affinity $Ca^{2+}$ proteins) | |
| Calreticulin | 25 |
| Grp 94/endoplasmin | 10 |
| Grp 78/BiP | binds $Ca^{2+}$ |
| PDI family | |
| PDI | 20 |
| ERp72 | 12 |
| ERCalcistorin/PDI | 20 |
| P5 | binds $Ca^{2+}$ |
| ERp57 | not investigated |
| ERp44 | not investigated |
| ERp29 | not investigated |
| Calsequestrin | 50 |
| **CLASS II proteins** (contain EF-hand $Ca^{2+}$ binding motifs and bind 6–7 moles of $Ca^{2+}$/mole of protein with high affinity) | |
| Reticulocalbin | |
| Cab45 | |
| Calumenin | |
| ERC55 | |
| Crocalbin/CBP-50 | |

$Ca^{2+}$ binding site, previously identified in many cytoplasmic proteins that bind $Ca^{2+}$ with high affinity.

A characteristic common to proteins in both classes is that they contain an N-terminal signal sequence that targets them to the lumen of the ER. They also terminate with a KDEL-like amino acid sequence that is responsible for their continual retrieval back to the ER lumen. Finally, most of the proteins resident in the lumen of the ER are multifunctional, regardless of their $Ca^{2+}$ binding properties. They are involved in maintaining $Ca^{2+}$ homeostasis and they assist in both protein folding and assembly, and lipid synthesis and transport.

## Class I $Ca^{2+}$ Binding Proteins in the ER Lumen

The class I ER luminal proteins are those that bind $Ca^{2+}$ with high capacity and low affinity. In most of these proteins, specific regions of the amino acid sequence contain a high proportion of acidic residues, and it is these acidic residues that are involved in the high capacity $Ca^{2+}$ binding. The following ER proteins belong to class I: calreticulin, Grp94, BiP, the PDI-family, and calsequestrin.

### Calreticulin

Calreticulin (46 kDa) is a major $Ca^{2+}$ storage protein in the ER lumen, which contains both high affinity/low capacity *and* low affinity/high capacity $Ca^{2+}$ binding sites. Specifically, it binds up to 25 moles of $Ca^{2+}$ per mole of protein with $K_d = 1$ mM (low affinity) and 1 mole of $Ca^{2+}$ per mole of protein with a $K_d = 1$ μM (high affinity). Calreticulin, one of the most extensively studied ER luminal proteins, is responsible for binding approximately 50% of the $Ca^{2+}$ stored in the lumen of the ER. As observed for other ER luminal proteins, the expression of calreticulin is up-regulated by stress, $Ca^{2+}$ depletion, and metabolic starvation. The overexpression of calreticulin in the ER significantly affects the ER $Ca^{2+}$ storage capacity, $Ca^{2+}$ uptake via SERCA, and release via $InsP_3$ receptor, indicating an important role for calreticulin in ER $Ca^{2+}$ buffering.

Calreticulin is composed of three structural and functional domains. The N-domain has the most conserved amino acid sequence and binds both $Zn^{2+}$ and ATP. In conjunction with the central, P-domain of the protein, it enables calreticulin's chaperone function. The central, P-domain of the protein is very rich in proline residues and contains several repeated amino acid sequences. It forms a highly unusual hairpin-like structure that involves the entire proline-rich region, and it is stabilized by a three-strand antiparallel B-sheet that is formed by the amino acid repeat sequences. The P-domain binds $Ca^{2+}$ with high affinity and low capacity. It also interacts with other ER chaperones and their substrates. The carboxyl-terminal C-domain of calreticulin contains a large number of acidic amino acid residues, and it is responsible for the high capacity $Ca^{2+}$ binding behavior of calreticulin.

In addition to binding and buffering $Ca^{2+}$ in the lumen of ER, calreticulin also plays a significant role as a molecular chaperone with a specificity for glycosylated proteins. Specifically, calreticulin binds N-linked, monoglucosylated carbohydrates on newly synthesized proteins. In conjunction with calnexin, an integral membrane protein and also a chaperone in the ER, calreticulin facilitates protein folding and oligomerization, and it supports quality control in protein folding processes. As components of the calreticulin/calnexin cycle, calreticulin might prefer secretory proteins and calnexin integral membrane proteins. Calreticulin and calnexin both form heterodimeric complexes with ERp57 and other chaperones. The formation of these chaperone–chaperone and chaperone–substrate interactions is regulated by changes in $Ca^{2+}$ concentration in the lumen of the ER.

### Grp94 (Glucose-Regulated Protein)

Grp94 (94 kDa) is another major $Ca^{2+}$ binding protein, and one of the most abundant proteins in the lumen

of the ER. It binds 8–10 mol of $Ca^{2+}$ per mol of protein with low affinity ($K_d = \sim 600\ \mu M$). Like calreticulin, Grp94 contains an acidic C-terminal region that comprises the low affinity $Ca^{2+}$ binding site. Because of their relatively high capacity for $Ca^{2+}$ binding and their abundance, Grp94 and calreticulin are the most significant $Ca^{2+}$ binding/buffering proteins in the ER lumen. The expression of Grp94 is up-regulated upon glucose starvation, hence its name (glucose-regulated protein). It binds ATP, but has only very low ATPase activity. It also functions as a molecular chaperone but binds to limited numbers of nascent proteins, most of them advanced folding intermediates or misfolded proteins that have been previously associated with other chaperones; it has a relatively low affinity for early folding intermediates. It appears that Grp94 is a chaperone specific for advanced intermediates in protein biosynthesis and that it works downstream of, or in conjunction with, other chaperones. $Ca^{2+}$ binding to Grp94 may play a role in the *in vivo* interactions of the protein with other chaperones and with its substrates.

## BiP (Immunoglobulin Binding Protein)

BiP is a 78-kDa ER chaperone that binds immunoglobulins. It is a monomeric protein with two distinct functional domains, an ATP-binding domain and a peptide-binding domain. While it has a relatively low capacity for binding $Ca^{2+}$ (1–2 mole of $Ca^{2+}$ per mole of protein), it contributes possibly as much as 25% of the total $Ca^{2+}$ storage capacity of the ER, and its elevated expression causes an appreciable increase in ER $Ca^{2+}$ storage capacity. BiP assists in the folding of newly synthesized polypeptides by binding to exposed hydrophobic side chains and subsequently coordinating the formation of their correct tertiary and quaternary structure. BiP binds ATP and has high ATPase activity essential for its chaperone function. Its association with nascent polypeptides is stabilized by the high concentrations of $Ca^{2+}$ in the ER lumen and likely involves $Ca^{2+}$ binding. The expression of BiP is up-regulated by a variety of stress conditions, including ER $Ca^{2+}$ depletion.

## The Protein Disulfide Isomerase (PDI) Family of Proteins

Several members of the protein disulfide isomerase (PDI) family are well-characterized. Each is folded similarly to thioredoxin, with a "typical" sequence of $\alpha$ helices and $\beta$ strands. PDI proteins also contain one or more active sites (typically two), again similar to that of thioredoxin. The main function of the PDI family of proteins is to catalyze the oxidation of disulfide bonds and to isomerize incorrectly formed disulfide bonds in newly synthesized polypeptides. Recent studies indicate that some members of this family (PDI, ERp72, and ERcalcistorin/PDI) also contribute to $Ca^{2+}$ buffering in the ER lumen.

### PDI

PDI is a 58-kDa $Ca^{2+}$-binding chaperone. It binds 19 mol of $Ca^{2+}$ per mol of protein with low affinity ($K_d = 2–5$ mM). The C-terminus of the protein contains numerous pairs of acidic residues that form the low affinity, high capacity $Ca^{2+}$ binding sites. PDI is an abundant and widely distributed ER luminal protein. Its major function is to catalyze disulfide bond formation in newly synthesized proteins and, importantly, high concentrations of $Ca^{2+}$ augment this activity. PDI contains a typical thioredoxin active site, which contains two cysteines separated by two other amino acid residues (the CXXC motif).

### ERp72

ERp72 is a 72-kDa member of the PDI family that binds 12 mol of $Ca^{2+}$ per mol protein with low affinity. Its amino acid sequence includes an acidic C-terminus that is involved in the high capacity $Ca^{2+}$ binding. ERp72 contains 3 thioredoxin-like active sites and has PDI-like activity. The expression of ERp72 is induced under stress conditions, including ER $Ca^{2+}$ depletion.

### ERcalcistorin/PDI

ERcalcistorin/PDI, a 58-kDa protein, also has high $Ca^{2+}$ binding capacity and low affinity. ERcalcistorin/PDI binds 23 mol of $Ca^{2+}$ per mol of protein with low affinity ($K_d = \sim 1$ mM). Like other class I ER $Ca^{2+}$ binding proteins, the high-capacity $Ca^{2+}$ binding sites in ERcalcistorin/PDI are localized to a C-terminal stretch of acidic amino acid residues. As noted previously for members of the PDI family, high concentrations of $Ca^{2+}$ augment the protein's isomerase activity. ERcalcistorin/PDI has 55% amino acid sequence identity to PDI, and it shows PDI-like chaperone activity.

### Other PDI Family Members

Other members of the PDI family have also been identified. While information regarding their $Ca^{2+}$ binding properties is limited, it has been established that they play a role in protein folding and posttranslational modification. P5, a 44-kDa polypeptide also known as CaBP1, contains two internal thioredoxin-like domains and a C-terminal KDEL retention/retrieval signal. While it does bind $Ca^{2+}$, it is unclear what role it plays in $Ca^{2+}$ buffering in the ER lumen. ERp57, a 57-kDa protein, carries out disulfide bond exchange

in conjunction/complex with calreticulin or calnexin. $Ca^{2+}$ binding to this protein has not been investigated. PDIR has the three CXXC motifs typically found in proteins within the PDI superfamily that are responsible for their oxidoreductase activity. PDIR is preferentially expressed in cells that actively secrete proteins, and its expression is stress-inducible. PDIp is a PDI-like protein expressed specifically in the pancreas. ERp44 and ERp29 are two other proteins of PDI family each containing a single thioredoxin-fold domain but no thioredoxin-like active site.

### Calsequestrin

Calsequestrin is a high-capacity $Ca^{2+}$ binding protein found in the SR of cardiac and skeletal muscle. It binds 40–50 moles of $Ca^{2+}$ per mole of protein with low affinity ($K_d = \sim 1–2$ mM). Small quantities of calsequestrin are also present in the ER of smooth muscle and the cerebellum. There are two isoforms of calsequestrin that are encoded by distinct genes: a 55-kDa cardiac form and 63-kDa skeletal muscle form. These proteins have very similar amino acid sequences with a C-terminal stretch containing over 100 acidic amino acids that are involved in the high-capacity $Ca^{2+}$ binding. Calsequestrin is localized to the terminal cisternae of the SR, where it acts as a $Ca^{2+}$ buffer. It lowers the concentration of free $Ca^{2+}$ inside the SR, thereby decreasing the concentration gradient against which the $Ca^{2+}$-ATPase must work as it transports $Ca^{2+}$ from the cytosol back into the SR. During the initiation of muscle contraction, $Ca^{2+}$ is released from the terminal cisternae of the SR into the cytosol via the ryanodine receptor. $Ca^{2+}$-induced conformational changes in calsequestrin affect the junctional face of these membrane proteins and this, in turn, regulates the opening and closing of the $Ca^{2+}$ channel. Interestingly, the crystal structure of calsequestrin shows that it contains three similar domains that contain a thioredoxin-like region. The thioredoxin-like fold may, therefore, be a common structural feature of ER/SR luminal proteins.

## Class II $Ca^{2+}$ Binding Proteins in the ER Lumen

Class II $Ca^{2+}$ binding proteins in the ER lumen are those that bind $Ca^{2+}$ with high affinity ($K_d = 1\ \mu M$ or less). Much less is known about this class of $Ca^{2+}$ binding proteins, which includes: reticulocalbin, Cab45, calumenin, ERC-55/TCBP-49/E6BP, crocalbin/CBP-50, and calreticulin. Calreticulin's structure, function, and $Ca^{2+}$ binding properties have been discussed already, with the class I ER proteins. The class II $Ca^{2+}$ binding proteins have also been found in the Golgi and may, therefore, play an important role in the secretory pathway. With the exception of calreticulin, they all contain six or seven EF-hand type, high-affinity $Ca^{2+}$ binding sites, and they are considered to be members of the reticulocalbin family. This family is a new subset of the EF-hand superfamily of proteins. The function of the high-affinity $Ca^{2+}$ binding sites in these proteins is not clear, but they may be involved in $Ca^{2+}$-dependent processes. They may also play an important structural role in the proteins.

Reticulocalbin is a 44-kDa protein that contains six EF-hand motifs. The difference between the amino acid sequences of these domains and EF-hand consensus sequences suggests that some of the domains may have lost their $Ca^{2+}$-binding capability. Cab45 is a 45-kDa, ubiquitously expressed protein. It contains six EF-hand $Ca^{2+}$ binding motifs. In contrast to reticulocalbin and ERC-55, which are soluble components of the ER, Cab45 is localized to the Golgi. Cab45 is the first calcium-binding protein that has been localized to the lumen of the Golgi, a post-ER compartment. Calumenin is a 39-kDa member of the reticulocalbin family. It is highly homologous to other family members, including reticulocalbin, Cab45, and ERC55. It is localized to the lumen of the ER and has a KDEL-like (HDEF) ER retrieval signal. ERC-55 (55 kDa) comprises an amino-terminal signal sequence followed by six copies of the EF-hand $Ca^{2+}$ binding motif.

## The Functional Significance of $Ca^{2+}$ Buffering in the ER

There are many $Ca^{2+}$-binding proteins in the ER lumen that both bind and buffer $Ca^{2+}$ and that also assist in protein folding and posttranslational modification. $Ca^{2+}$ buffering within the ER lumen is an essential component of proper cellular $Ca^{2+}$ homeostasis. Furthermore, it has a profound effect on other ER functions including protein and lipid synthesis and stress response. For example, reduction of $Ca^{2+}$ concentration in the ER leads to the accumulation of misfolded proteins, increased expression of ER chaperones, and ER to nucleus/ER to plasma membrane signaling, which inhibits protein synthesis and facilitates protein degradation. Overall, disruption of ER $Ca^{2+}$ homeostasis has profound effects on intracellular communication and cell growth, resulting in organellar diseases, and it has detrimental effects at cellular and systemic levels.

### See Also the Following Articles

Calcium-Binding Proteins: Cytosolic (Annexins, Gelsolins, $C_2$-Domain Proteins) • Calcium-Modulated Proteins (EF-hand) • Chaperones, Molecular

## Glossary

**calreticulin** Multifunctional $Ca^{2+}$ binding/buffering ER resident chaperone. The protein is responsible for the buffering of over 50% of ER luminal $Ca^{2+}$ and for assisting in the folding of newly synthesized glycoproteins.

**chaperones** Molecules that bind to misfolded proteins and assist in their folding and posttranslational modification.

**endoplasmic reticulum** Intracellular organelle involved in many cellular functions including modulation of $Ca^{2+}$ homeostasis, protein synthesis and modification, and lipid synthesis.

**protein disulfide isomerase** Belonging to a family of proteins containing thioredoxin, they modify and catalyze the formation of disulfide bonds in newly synthesized proteins.

## Further Reading

Baumann, O., and Walz, B. (2001). Endoplasmic reticulum of animal cells and its organization into structural and functional domains. *Int. Rev. Cytol.* **205**, 149–214.

Eggleton, P., and Michalak, M. (2003). *Calreticulin in Health and Disease*. R. G. Landes Co., Austin, Texas.

Molinari, M., and Helenius, A. (2000). Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* **288**, 331–333.

Noiva, R. (1999). Protein disulfide isomerase: The multifunctional redox chaperone of the endoplasmic reticulum. *Sem. Cell Dev. Biol.* **10**, 481–493.

## Biography

Jody Groenendyk is a graduate student in the Department of Pediatrics, Faculty of Medicine, and the University of Alberta. For several years she has been involved in studies on $Ca^{2+}$ and buffering proteins for the ER. She is supported by the Alberta Heritage Foundation for Medical Research and by the Canadian Institutes for Health Research.

Marek Michalak is a Professor of Biochemistry at the University of Alberta, Edmonton, Alberta. He obtained his Ph.D. at the Nencki Institute for Experimental Biology, Warsaw, Poland. Michalak's laboratory has been studying ER luminal proteins for almost 20 years. The major focus of this work is on $Ca^{2+}$ buffering and chaperone protein, with a special emphasis on calreticulin. He has published over 150 papers in the area of ER $Ca^{2+}$ binding chaperones. He has authored and edited books on the structure and function of calreticulin and calnexin.

# Calcium Oscillations

**Marisa Brini**
*University of Padova and Venetian Institute of Molecular Medicine (VIMM), Padova, Italy*

**Cellular signaling mechanisms are designed to transmit information from the cell surface to specific targets within the cell. Usually, the information is transmitted by intracellular messengers, of which the calcium ion, $Ca^{2+}$, is one of the most important. Generally, extracellular stimuli are converted in a transient increase in the cytosolic $Ca^{2+}$ concentration, $[Ca^{2+}]_c$, which, in turn, activates cellular functions. The specificity of the activated signal is guaranteed by the complex spatial and temporal organization of the changes in $Ca^{2+}$ concentration, which increases the versatility of this messenger. Different cell types use distinct $Ca^{2+}$ signals, as appropriate to their physiology. The ability to use $Ca^{2+}$ in different modes helps cells to vary the amplitude, frequency, kinetics, and localization of the signal. It is now recognized that periodic $[Ca^{2+}]_c$ changes (i.e., oscillations) probably represent the typical response of cells to physiological agonists concentrations.**

## General Considerations

It has been known since the 1950s that the periodic opening of plasma-membrane $Ca^{2+}$ channels, such as induced, for example, by the rhythmic changes of the plasma membrane potential of the heart or by burst of action potential in neurons, can produce fluctuations in cytosolic $[Ca^{2+}]$. The idea that $[Ca^{2+}]_c$ oscillations may also occur in nonexcitable cells has emerged much later, thanks to seminal observations by Cobbold and coworkers in the mid-1980s on the fertilization of oocytes and on hormone-stimulated hepatocytes. Surprisingly, these oscillations were not dependent on the periodic opening and closing of plasma membrane $Ca^{2+}$ channels, but rather on cycles of $Ca^{2+}$ release and uptake from the intracellular compartment sensitive to the second messengers inositol (1,4,5)-trisphosphate ($InsP_3$), which is the endoplasmic reticulum. Later, $Ca^{2+}$ oscillations were observed in other animal as well as plant cells, even if many of these cells do not have an obvious oscillatory biological function.

A question that immediately arises is the reason for the transmission of the $Ca^{2+}$ signal by oscillations rather than by a stationary change in $Ca^{2+}$ concentration. It is generally assumed that the oscillatory behavior has physiological advantages. This is so because the elevation of $Ca^{2+}$ concentration over a long period is known to be lethal to the cell: the oscillatory behavior would prevent this negative effect, while still permitting optimal activation of $Ca^{2+}$ sensing enzymes. The oscillatory regime would also prevent long-lasting receptor desensitization thus increasing the sensitivity of sensing enzymes to $Ca^{2+}$, i.e., $[Ca^{2+}]$ would be permitted to periodically exceed the threshold for enzyme activation, even its level would remain below the threshold. Another advantage is the wide range of temporal and spatial aspects that oscillations can assume, allowing different signals to be transmitted within cells and from cell to cell.

Usually, $Ca^{2+}$ oscillations are characterized by rather constant amplitude and by a variable frequency, which ranges from 5 to 60 s depending to the cell type, and on the nature and the strength of the stimulus (Figure 1). These characteristics have led to the concept of frequency-modulated $Ca^{2+}$ signaling, which reflects the ability of cells to interpret changes in the frequency of the oscillations.

## Mechanism of $[Ca^{2+}]_c$ Oscillations

The mechanism of $Ca^{2+}$ oscillation has received much experimental and theoretical attention, but is still largely debated. A first distinction considers the origin of the $Ca^{2+}$ spikes, for which two major mechanisms exist: one is linked to the periodic opening of plasma membrane channels and another to cycles of $Ca^{2+}$ release and uptake from intracellular stores (Figure 2). However, this distinction is incomplete since very often the contributions of intracellular $Ca^{2+}$ release and of extracellular $Ca^{2+}$ influx cannot be clearly separated. An obvious example is that of cardiac myocytes, where the periodic contraction of the cells is directly promoted by the release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR), which is triggered by localized influx of $Ca^{2+}$ through plasma-membrane voltage-operated $Ca^{2+}$ channels (VOCs), a process known as $Ca^{2+}$-induced $Ca^{2+}$ release (CIRC).

In excitable cells such as neurons, heart, and neuroendocrine cells, the transient $[Ca^{2+}]$ elevation is

 © 2004, Elsevier Inc. All Rights Reserved. 

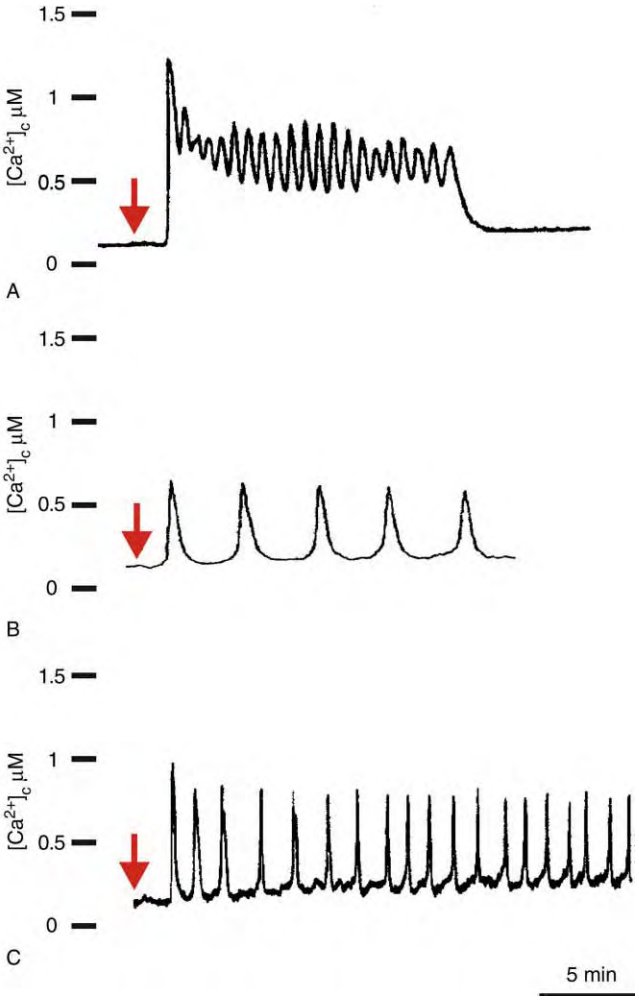



**FIGURE 1** Examples of different oscillatory pathways. A, parotid gland; B, hepatocyte; C, endothelial cell. The pattern of oscillations may vary with respect to amplitude and period. The red arrow indicates agonist stimulation.

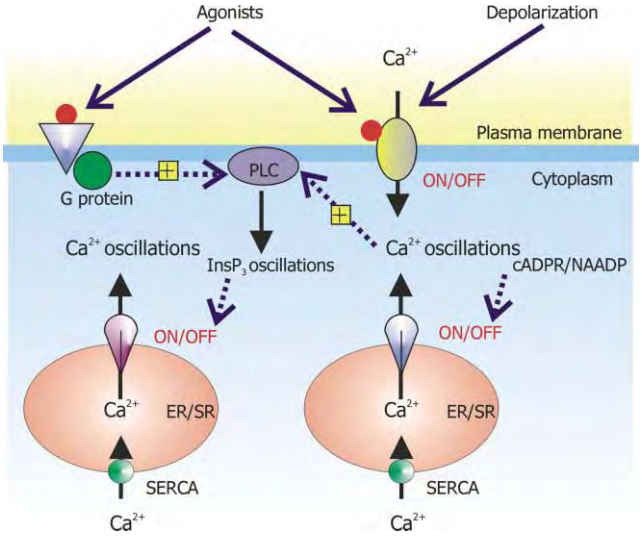



**FIGURE 2** Signaling pathways that participate in the generation of $Ca^{2+}$ oscillations. Three models are proposed. In excitable cells $Ca^{2+}$ oscillations are evoked by rhythmic oscillatory changes in membrane potential. In nonexcitable cells they are induced either by the oscillatory production of $InsP_3$, or following the oscillatory inactivation of $InsP_3$ receptors (ON/OFF). ER/SR, endoplasmic/sarcoplasmic reticulum; PLC, phospholipase C; SERCA, sarco/endoplasmic reticulum calcium ATPase; $InsP_3$, inositol 1,4,5-trisphosphate; cADPR, cyclic ADP-ribose; NAADP, nicotinic acid adenine dinucleotide phosphate.

due to $Ca^{2+}$ entry through voltage-operated $Ca^{2+}$ channels or receptor-operated $Ca^{2+}$ channels (ROCs) activated in response to neurotransmitters. In these cells the oscillatory behavior is secondary to the oscillatory nature of the trigger itself. For example, cortical and cerebellar neurons in primary cultures exhibit spontaneous oscillations that occur in synchrony with adjacent neurons and are dependent on synaptic activity, i.e., changes of membrane potential.

In nonexcitable cells the predominant mechanism of $[Ca^{2+}]_c$ elevation is through the activation of plasma membrane receptors coupled to G proteins and the phosphoinositide pathway. Activation of membrane phospholipase C (PLC) generates the second messenger $InsP_3$ from phosphatidylinositol 4,5-bisphosphate, mobilizing $Ca^{2+}$ from stores, associated with the endoplasmic or sarcoplasmic reticulum. The pattern of the $Ca^{2+}$ signals is often complex but $InsP_3$-linked agonists frequently give rise to repetitive $[Ca^{2+}]_c$ transients.

Other molecules (i.e., cyclic ADP-ribose, cADPR, and nicotinic acid adenine dinucleotide phosphate, NAADP) have recently also been shown to mobilize $Ca^{2+}$ from internal stores generating $Ca^{2+}$ oscillations. Studies using $Ca^{2+}$ indicator dyes targeted to the lumen of intracellular stores of intact cells have shown that agonist-induced cytosolic $Ca^{2+}$ oscillations are accompanied by inverse oscillations of stored $Ca^{2+}$.

Recent works suggest at least two possible mechanisms for the generation of oscillatory $Ca^{2+}$ signals: either an oscillatory production of $InsP_3$ or oscillatory inactivation of $InsP_3$ receptors. Both mechanisms appear to operate in different cell types, the common denominator being the positive and negative feedback by $Ca^{2+}$ on the release system, e.g., in hormone-stimulated hepatocytes, oscillations are driven by the cycling of the $InsP_3$ channels between a fully open and a largely closed state, rather than by oscillations in $InsP_3$. Similarly, in pancreatic acinar cells, the pulsatile calcium release does not depend on fluctuations in $InsP_3$ concentration, but in kidney epithelial cells spatiotemporal changes in the concentration of $InsP_3$ appear to be synchronous with $Ca^{2+}$ oscillations, and intracellular $InsP_3$ waves accompany $Ca^{2+}$ waves.

The fluctuations in $InsP_3$ may be controlled by $Ca^{2+}$ itself through the regulation of PLC activity or through

regulatory proteins which act directly on G proteins, thus affecting the downstream $InsP_3$ production. Alternatively, the activity of the $InsP_3$-sensitive $Ca^{2+}$ channels may be controlled by $Ca^{2+}$ itself both at the cytosolic and the luminal side, and, possibly, by kinase-mediated phosphorylation/dephosphorylation cycles. Thus, low concentrations of cytosolic $Ca^{2+}$ increase the open probability of the channels, whereas higher concentrations favor their closure, generating the oscillatory $Ca^{2+}$-signaling pattern.

## Role and Functional Significance of $[Ca^{2+}]_c$ Oscillations

$Ca^{2+}$ oscillations have been implicated in the control of different cell processes, among them oocyte activation and fertilization, growth-cone migration and tuning, axonal growth of cortical neurons, neuronal-cell migration, development of neurotransmitter phenotypes, formation of nodules in plant root hairs, developmental of muscle, release of cytokines from renal epithelial cells, and disassembly of adhesive structures during cell migration (Figure 3).

Numerous experimental protocols modulate the amplitude and the frequency of $Ca^{2+}$ spikes in living cells. In general, cells choose the shape of $Ca^{2+}$ signals according to the type of process that must be activated: single $Ca^{2+}$ transients are used to activate processes such as muscle contraction, repetitive signals are used when information must be relayed over long time periods, as in fertilization and in the triggering of the developmental program. Furthermore, oscillations can be directed to discrete subcellular domains to generate large local $[Ca^{2+}]_c$ increases required to couple $[Ca^{2+}]_c$ to secretion and to mitochondrial metabolism. They can also propagate through entire cells, or even through coupled cells to coordinate the activities in intact tissues and organs.

The most obvious mechanism for the regulating of specific functions would seem to be modulation of the amplitude of $Ca^{2+}$ signals. However, the efficiency of the activating stimulus is frequently related to the frequency rather than to the amplitude of the $[Ca^{2+}]_c$ oscillations.

A particularly illustrative example of decoding of the $Ca^{2+}$ signal through the frequency of the oscillations is the $Ca^{2+}$-regulated gene expression.

Dolmetsch and colleagues have recently reported that $Ca^{2+}$ oscillations in T-lymphocytes reduce the effective $Ca^{2+}$-threshold for activating some transcription factors, more effectively than the sustained $Ca^{2+}$ increase. The efficacy is encoded by the oscillation frequency: rapid oscillations stimulate three transcription factors (NF-AT, Oct/OAP, and NF-κB), while lower-frequency oscillations only activate NF-κB. Thus, $Ca^{2+}$-modulated gene transcription is a frequency-sensitive process. Another elegant example of the correlation between the frequency of the $[Ca^{2+}]$ spikes and cellular function is the translation of repetitive cytosolic $Ca^{2+}$ spiking through mitochondrial $Ca^{2+}$ oscillations in primary cultures of rat hepatocytes in the final and sustained elevation of NAD(P)H production. This coupling allows the coordination of mitochondrial ATP production, linked to the sustained activation of mitochondrial $Ca^{2+}$-sensitive dehydrogenases, with the level of energy demand, at the same time avoiding the deleterious effects of the prolonged increase in $[Ca^{2+}]$.

In polarized cells, such as those of pancreatic acini, the receptor-evoked $Ca^{2+}$ signal initiates at the apical pole, generating agonist-specific $Ca^{2+}$ oscillation patterns. Acetylcholine stimulation evokes repetitive local spikes, which remain confined to the secretory apical pole. Cholecystokin instead induces local $Ca^{2+}$ spikes

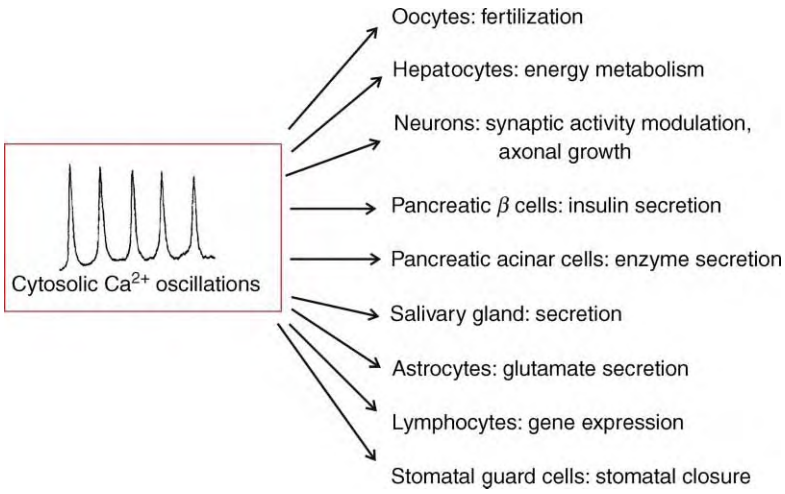



**FIGURE 3** Scheme of the main cellular events modulated by the oscillatory behavior of $Ca^{2+}$ signal.

that end in longer-lasting $Ca^{2+}$ transients that spread to the whole cell.

Last but not least, in considering the functional significance of $Ca^{2+}$ oscillations it is necessary to empathize their role in the central nervous system. In neurons, the sequential openings of plasma membrane $Ca^{2+}$ channels lead to rhythmic $Ca^{2+}$ spikes that, in turn, are linked to synaptic communication within and among neuronal circuitries, i.e., by inducing the release of neurotransmitters. A peculiar role has recently been attributed to the spontaneous and synchronous $Ca^{2+}$ oscillations that occur independently from synaptic activity in developing neurons, such as those in rat visual cortex. It was suggested that these oscillations could propagate through different cells and determine a common developmental pattern of cells from the same domain.

A sophisticated example of the role of $Ca^{2+}$ oscillation in the coordination of the activity of neuronal circuits has been described in glial astrocytes, a cell type of the central nervous system, which plays a number of important functions. Very recently, it has been proposed that they work as detectors of synaptic activity: by changing the frequency of $Ca^{2+}$ oscillations evoked by the synaptic release of glutamate, these astrocytes acquire the ability to discriminate between different levels and patterns of synaptic activity.

## How Are $[Ca^{2+}]_c$ Oscillations Decoded?

Although the frequency of $Ca^{2+}$ oscillations seems critical for the induction of selective cellular functions, the mechanisms by which the cell decodes the oscillatory signal remain unclear.

In 1998 De Koninck and Schulman showed that the oscillation frequency modulates the activity of $Ca^{2+}$-calmodulin Kinase II (CaMKII) *in vitro*. This enzyme is tuned to respond optimally to $Ca^{2+}$ oscillations with high frequency. The multimeric structure of the enzyme, coupled to its autophosphorylation properties, has led to the suggestion that it can act as a frequency decoder of $[Ca^{2+}]_c$ oscillations. The enzyme is now considered of special importance to the nervous system, where it regulates the process of memory formation and storage.

More recently, the advent of the green fluorescent protein (GFP) technology has shown that protein kinase C (PKC), one of the most important enzymes in cell signaling, undergoes an oscillatory plasma membrane association in phase with receptor-mediated oscillations in $[Ca^{2+}]_c$. Each $[Ca^{2+}]_c$ oscillation induces the transient membrane association of PKC leading to a transient burst of PKC substrate phosphorylation. It was suggested that the detachment of PKC from the membrane, which occurs as $Ca^{2+}$ returns to the basal level between oscillations, results in a pause of PKC activity, allowing substrate dephosphorylation. In this respect, the $Ca^{2+}$-mediated regulation of PKC differs from that of CaMKII, since the latter does not fully deactivate between spikes as the frequency of $[Ca^{2+}]_c$ oscillations increases.

## See Also the Following Articles

Calcium Signaling: Cell Cycle • Ligand-Operated Membrane Channels: Calcium (Glutamate) • Phosphatidylinositol Bisphosphate and Trisphosphate • Phospholipase C • Store-Operated Membrane Channels: Calcium • Voltage-Sensitive $Ca^{2+}$ Channels

## Glossary

**G protein** Protein associated to specific hormone receptor of the plasma membrane and responsible for the conversion of extracellular signals in intracellular messages.

**inositol (1,4,5)-trisphosphate ($InsP_3$)** Diffusible cytosolic messenger which induces the release of $Ca^{2+}$ from intracellular stores (the endo/sarcoplasmic reticulum) by opening $InsP_3$-sensitive $Ca^{2+}$ channels.

**membrane potential** Electrical gradient across the plasma membrane (and other membranes as well).

**NAD(P)H** Nicotinamide adenine dinucleotide, acceptor of the reducing equivalent produced in the oxidation of most metabolites. It is a small organic molecule, which participates as a coenzyme in enzymatic reactions.

**neurotransmitters** Chemical messengers by which neurons communicate with each other.

## Further Reading

Berridge, M. J. (1990). Calcium oscillations. *J. Biol. Chem.* **265**, 9583–9586.

Berridge, M. J., and Dupont, G. (1994). Spatial and temporal signalling by calcium. *Curr. Opin. Cell Biol.* **6**, 267–274.

Carafoli, E., Santella, L., Branca, D., and Brini, M. (2001). Generation, control, and processing of cellular calcium signals. *Crit. Rev. Biochem. Mol. Biol.* **36**, 107–260.

Petersen, O. H., Petersen, C. C., and Kasai, H. (1994). Calcium and hormone action. *Annu. Rev. Physiol.* **56**, 297–319.

Thomas, A. P., St. Bird, G. J., Hajnóczky, G., Robb-Gaspers, L. D., and Putney, J. W., Jr. (1996). Spatial and temporal aspects of cellular calcium signalling. *FASEB J.* **10**, 1505–1517.

## Biography

Marisa Brini is an Assistant Professor of Biochemistry at the University of Padova. Her main interest is in the study of calcium signaling defects occurring in human genetic diseases and/or transgenic animal models (malignant hyperthermia, mitochondrial disorders, and various cardiopathies) in which the homeostasis of $Ca^{2+}$ in muscle is altered.

# Calcium Sensing Receptor

**Jacob Tfelt-Hansen and Edward M. Brown**
*Brigham and Women's Hospital, Harvard University, Boston, Massachusetts, USA*

**The calcium sensing receptor (CaR) is a membrane-bound, G protein-coupled receptor that is expressed in all tissues that regulate extracellular calcium homeostasis – the parathyroid glands, thyroidal C cells, bone, kidney, and intestine. The CaR senses the level of calcium in the blood and acts, therefore, as the body's thermostat for calcium (or calciostat). When the level of calcium changes by even a few percent from its normal level, the CaR senses this change and then modulates the functions of the cells expressing it so as to restore the level of blood calcium to normal. The CaR-mediated regulation of the secretion of parathyroid hormone plays a particularly important role in calcium homeostasis because it directly or indirectly modulates the functions of all tissues involved in regulating blood calcium.**

## Background

The body maintains the level of extracellular calcium ($Ca_o^{2+}$) within a narrow range (1.1–1.3 mM) because both high and low levels of $Ca_o^{2+}$ are dangerous and can be life-threatening. Very small changes in $Ca_o^{2+}$, on the order of a few percent, lead to immediate physiological responses that restore the level of $Ca_o^{2+}$ to normal. These fast responses are essential because rapid changes in $Ca_o^{2+}$ are more dangerous than those that develop more slowly. The calcium sensing receptor (CaR), which was cloned almost a decade ago, plays a central role in this delicate homeostatic system. The CaR acts like a thermostat, but instead of measuring changes in temperature it measures alterations in the level of $Ca_o^{2+}$, thereby functioning as a calciostat. This means that the CaR tells the chief cells of the parathyroid glands the exact level of $Ca_o^{2+}$. The CaR is a seven-transmembrane-domain receptor that is coupled to G proteins – called a G protein-coupled receptor (GPCR). The importance of the CaR is illustrated by the diseases caused by naturally occurring mutations in it that lead to either loss of function or gain of function. Heterozygous (one-allele) loss-of-function mutations give rise to familial hypocalciuric hypercalcemia (FHH, also called familial benign hypocalciuric hypercalcemia, FBHH), in which most patients have asymptomatic hypercalcemia. The homozygous variant of inactivating CaR mutations, in contrast, is a much more severe, potentially fatal disease if left untreated; it is called neonatal severe primary hyperparathyroidism (NSHPT). The mouse model of FHH (e.g., heterozygous knockout of the CaR gene) has a phenotype comparable to that of the human condition, suggesting that the number of receptors on the cell surface is the determining factor for the clinical expression of the disease. Gain-of-function mutations give rise to autosomal-dominant hypoparathyroidism (ADH). Most patients with this condition have asymptomatic hypocalcemia. Although the principal role for the CaR is in calcium homeostasis, other functions of the receptor have been elucidated in the wide variety of cell types expressing it. The CaR can modulate the cell cycle, regulate ion channels, and control peptide secretion. This entry provides a broad overview of the CaR's biochemical features, a discussion of its functions in normal and pathophysiologic states, and a discussion of new drugs that have been developed that target the CaR.

## Biochemical Features of the CaR

The CaR is a seven-transmembrane-domain GPCR whose gene is located on chromosome 3q. Other receptors in the superfamily of GPCRs that are related structurally to the CaR include the $GABA_B$ and metabotropic glutamate receptors (mGluRs), which recognize gamma aminobutyric acid (GABA) and glutamate, respectively, as their ligands, as well as putative pheromone and odorant receptors.

### Structure

The human CaR has a very large N-terminal extracellular domain (ECD) of 612 amino acids (Figure 1). The ECD contains binding sites for calcium and is thought to have a bilobed venus flytrap structure that closes upon binding its principal physiological ligand, extracellular calcium ions. The binding of $Ca_o^{2+}$ is very weak compared to many other receptors. These weak forces between the ECD and $Ca_o^{2+}$ are crucial for the receptor to act as a calciostat. The CaR has a central core of

 © 2004, Elsevier Inc. All Rights Reserved. 

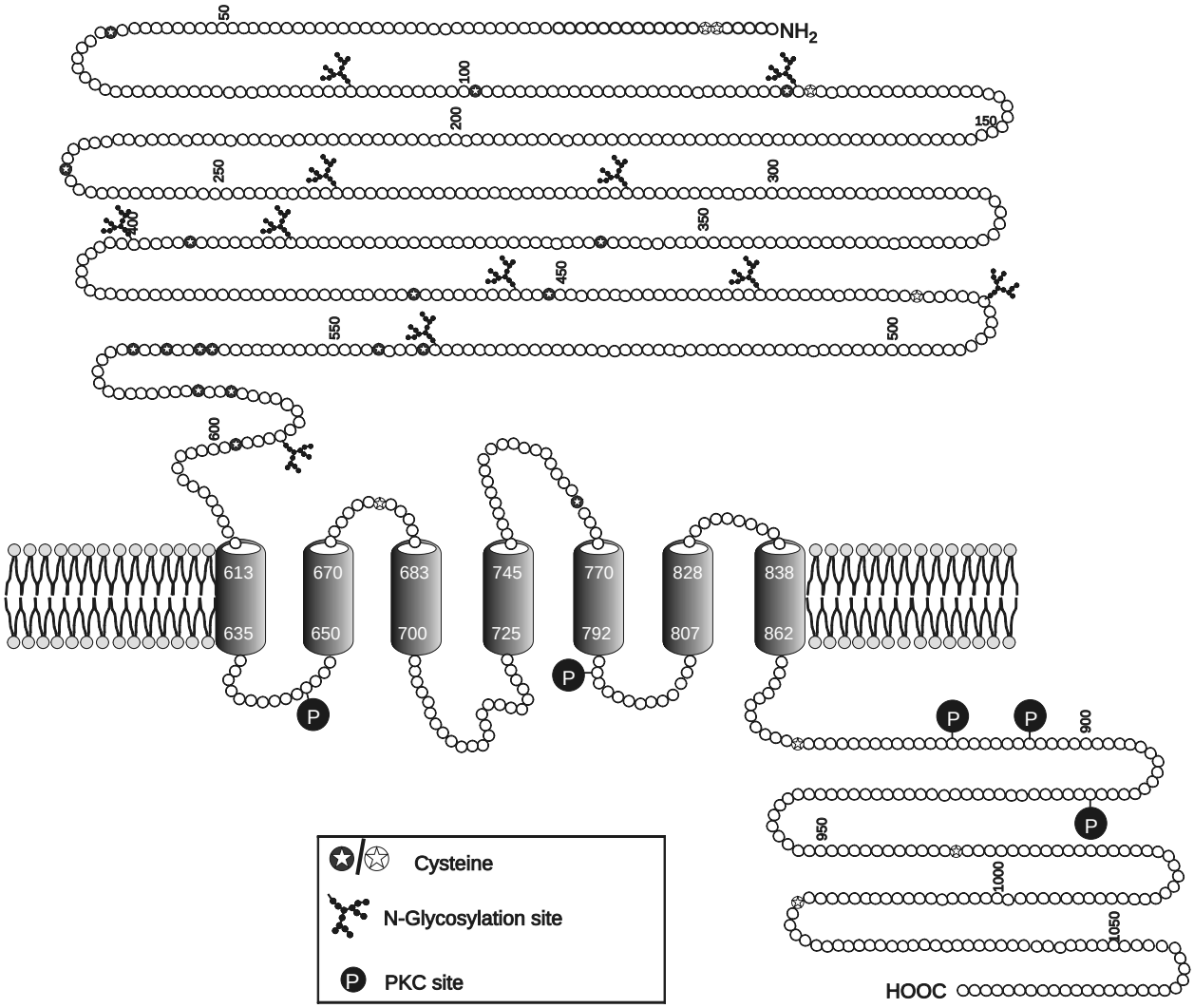



**FIGURE 1** Representation of the predicted topological features of the human extracellular $Ca^{2+}$ sensing receptor. The extracellular domain contains 612 amino acids, and the transmembrane domains and intracellular C tail each contain approximately 200 amino acids. Also shown are the PKC sites, N-glycosylation sites, and cysteine residues conserved with the metatropic glutamate receptors.

250 amino acids with seven transmembrane domains (TMDs), which is characteristic of the GPCRs. The TMDs serve as the target for newly developed drugs (discussed later). The intracellular carboxy (C) terminus of the receptor consists of 216 amino acids and has an important role, along with the CaR's intracellular loops, in the transduction of the $Ca_o^{2+}$ signal to inside the cell.

### N-Glycosylation with Carbohydrates is Crucial for Cell Surface Expression

The CaR has several N-linked glycosylation sites that are important for its expression on the cell surface. Western blotting of the CaR shows several bands between 120 and 200 kDa. A minor band at 120 kDa represents the nonglycosylated CaR, whereas major bands at 140 and 160 kDa correspond to the immature, high mannose and mature, fully N-glycosylated forms of the CaR, respectively. If the degree of glycosylation of the receptor is reduced to a significant extent by site-directed mutagenesis of its N-linked glycosylation sites, less of the receptor reaches the cell surface.

### CaR Functions as a Dimer on the Cell Surface

Additional immunoreactive bands observed on Western blot analysis of the CaR at $>200$ kDa represent CaR dimers or even larger species. The dimer is the major form of CaR on the cell surface and is thought to be the active form of the receptor. Two monomers of the CaR are linked covalently by two disulfide bonds between cysteine residues 129 and 131 as well as by noncovalent interactions. The disulfide bonds constrain the receptor

in its inactive form; disrupting them produces a receptor that responds to lower than normal levels of $Ca_o^{2+}$. Moreover, heterodimerization of a normal CaR monomer with a monomer harboring an FHH mutation can interfere with the function of the wild-type CaR monomer, documenting that there are functional interactions between the monomers of the dimeric CaR.

### Amino Acids in Intracellular Loops are Pivotal for Activation of Intracellular Signaling by the CaR

Amino acids in intracellular loops 2 and 3 are key players in determining selective coupling of the CaR to G proteins. For example, two amino acids residues within intracellular loop 2 and eight residues in intracellular loop 3 have been shown to be important for the G protein-dependent activation of phospholipase C (PLC) – one of the major intracellular signaling pathways used by the CaR.

### The C Tail of the CaR is Important for its Function

The amino acid sequence of the proximal portion of the C tail of the CaR is highly conserved among species, suggesting its functional importance. In addition, naturally occurring mutations in the C tail have been shown to cause the diseases mentioned earlier (e.g., FHH and ADH). Furthermore, certain truncation and deletion mutations of the receptor's C tail up-regulate the expression of the receptor on the cell surface, thereby causing gain of function. Other truncations have been shown to cause loss of function, demonstrating that there are discrete elements within the C tail that modulate both the cell surface expression and function of the receptor.

### Functionally Important Mutations in the CaR as Experiments in Nature

Approximately 100 naturally occurring mutations have so far been described that cause either a loss of function of the CaR in FHH and NSHPT or a gain of function in ADH. Deletion, nonsense, insertion, missense, truncations, and splice-site mutations have been described throughout the whole CaR gene. They have not only proven the physiological importance of the CaR in $Ca_o^{2+}$ homeostasis, but have also provided an important source of information about the structure-function relationships of the receptor, as noted previously.

## Agonists of the CaR

As previously mentioned, the CaR can detect very small changes in the level of $Ca_{o,}^{2+}$ on the order of a few percent. The receptor's principal physiological agonist, $Ca_o^{2+}$, regulates its activity at millimolar concentrations, implying an extremely low affinity compared with those of other receptors for their respective agonists. Calcium has been shown to interact with the ECD of the CaR. This has been established elegantly through the creation of a chimeric receptor containing the ECD of the CaR and the TMDs and C tail of an mGluR. The chimeric receptor responds to $Ca_o^{2+}$ but not to glutamate. The CaR's Hill coefficient is typically 3–4, implying the presence of several binding sites for $Ca_o^{2+}$, but the steepness of the physiological response of the parathyroid glands to $Ca^{2+}$ is even more extreme. Suppression of parathyroid hormone (PTH) release *in vitro* is minimal at 0.75 mM and maximal at ~2 mM. Unlike many receptors, the CaR is resistant to desensitization; this optimizes the CaR's capacity for continuous surveillance of extracellular $Ca^{2+}$ levels. Although $Ca_o^{2+}$ is the major ligand for the CaR, a variety of other agonists have been identified. $Mg^{2+}$(another biologically important divalent cation whose extracellular level is maintained within a narrow range) has been shown, like $Ca_o^{2+}$, to inhibit PTH secretion. Although this effect seems to be at supraphysiological levels, magnesium's action on the CaR in the kidney may regulate its renal reabsorption and contribute to $Mg_o^{2+}$ homeostasis. Other well-established agonists acting on the ECD are gadalinium, neomycin, and spermine; all exhibit positive cooperativity with regard to their interactions with calcium in activating the receptor. These three agonists, as well as $Mg_o^{2+}$, are defined as type 1 agonists, which bind to the ECD and activate the CaR regardless of whether $Ca_o^{2+}$ is present. Type 2 agonists, in contrast, are allosteric activators that require calcium to exert their effects. Certain L-amino acids, particularly aromatic amino acids, have been shown to be type 2 agonists, binding to a site within the ECD of the CaR. It is possible that they function in this manner in the gut, where the CaR is widely distributed, enabling the CaR to act, in effect, as a nutrient receptor. The CaR also senses ionic strength, as illustrated by the fact that exposing dispersed bovine parathyroid cells to a 40-mM increment in NaCl shifts the $EC_{50}$ for high $Ca_o^{2+}$-evoked inhibition of PTH release by at least 0.5 mM. Ionic strength may exert physiologically relevant actions on the CaR in select regions of the gastrointestinal (GI) tract and kidney, where the levels of ionic strength can vary greatly.

## Intracellular Signaling Pathways Used by the CaR

CaR agonists activate phospholipases C, $A_2$, and D in parathyroid as well as in CaR-transfected but not in nontransfected human embryonic kidney (HEK293) cells. The high $Ca_o^{2+}$-evoked, transient rise in the cytosolic calcium concentration ($Ca_i^{2+}$) in bovine parathyroid and CaR-transfected HEK293 cells is mediated

by activation of PLC and the resultant inositol triphosphate ($IP_3$)-mediated release of $Ca^{2+}$ from intracellular stores. In parathyroid and CaR-transfected HEK293 cells, the activation of PLC is insensitive to pertussis toxin and probably involves $G\alpha_{q/11}$. In contrast, the CaR-mediated increase in inositol phosphates observed in the mouse pituitary AtT-20 cell line is sensitive to pertussis toxin, showing that the CaR can also activate PLC through pertussis toxin-sensitive G proteins (i.e., $G\alpha_i$). The CaR also stimulates the p42/44 mitogen-activated protein kinases (MAPKs) by a pathway that involves cytoplasmic tyrosine kinases (e.g., c-Src) and inhibits adenylate cyclase via $G\alpha_i$ in parathyroid and some kidney cells. In other cells, the CaR inhibits the accumulation of cAMP by increasing $Ca_i^{2+}$, which then inhibits the activity of a $Ca^{2+}$-inhibitable isoform of adenylate cyclase.

### Feedback Regulation of the CaR by Protein Kinase C

The CaR has two protein kinase A (PKA) and five protein kinase C (PKC) phosphorylation sites within its intracellular domains. Phosphorylation of these PKC sites, especially the one located at threonine 888, inhibits the receptor's coupling to PLC, one of the CaR's principal intracellular signaling pathways. This serves as a negative feedback loop because PKC is activated downstream of PLC.

## The Importance of the CaR in Calcium Homeostasis

The level of calcium is regulated by three calciotropic hormones: PTH, calcitonin (CT), and $1,25(OH)_2$ vitamin $D_3$. There is an inverse sigmoidal relationship between PTH, a key calcium-elevating hormone, and $Ca_o^{2+}$ (Figure 2), whereas there is a positive relationship between $Ca_o^{2+}$ and CT, the calcium-lowering hormone, both of which are mediated by the CaR. These features confer on the CaR the role of the gatekeeper to normal calcium homeostasis. The additional $Ca_o^{2+}$-elevating hormone, $1,25(OH)_2$ vitamin $D_3$, like PTH, displays an inverse relationship with $Ca_o^{2+}$, although whether this is CaR-mediated is currently unknown. In addition to the effects of the CaR on calciotropic hormone production and secretion, the receptor also directly modulates the function of target tissues for PTH, especially the kidney and perhaps in bone and intestine. In this way, $Ca_o^{2+}$ functions as a first messenger, serving as the body's major $Ca_o^{2+}$-lowering hormone (described later).

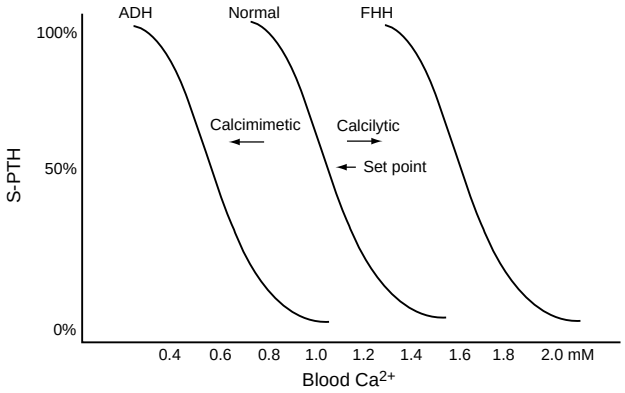



**FIGURE 2** Sigmoidal relationship between the blood $Ca^{2+}$ and serum PTH (S-PTH) levels in normal individuals (eucalcemia), in autosomal dominant hypoparathyroidism (ADH) (hypocalcemia), and in familial hypocalciuric hypercalcemia (FHH). The arrows represent the effects of calcimimetics (shifting the set point to the left) and calcilytics (shifting the set point to the right). Set point is defined as the calcium level that produces one-half of the maximal inhibition of the PTH secretion.

## CaR in Hypercalcemia

The responses of the CaR to hypercalcemia provide an accurate picture of its role in calcium homeostasis. An increase in $Ca_o^{2+}$ is sensed by the chief cells in the parathyroid gland, which within seconds lowers the rate of PTH secretion. The impact of this reduction in PTH secretion and in the direct actions of $Ca_o^{2+}$ on cellular function occur in several target tissues.

### Bone

The high $Ca_o^{2+}$-elicited lowering of PTH decreases the rate of release of calcium ions from bone – an action mediated through its action on the PTH receptor (PTHR) on the osteoblast. The production of $1,25(OH)_2$ vitamin $D_3$ is likewise inhibited by hypercalcemia – and this fall in $1,25(OH)_2$ vitamin $D_3$ also reduces bone resorption. High $Ca_o^{2+}$, by itself, also exerts negative effects on osteoclast formation and function and increases osteoblast activity, thereby reducing the net release of calcium from bone. Taken together, the effects of PTH, $1,25(OH)_2$ vitamin $D_3$, and $Ca_o^{2+}$ on bone all lower the level of blood calcium.

### Kidney and Intestine

The high $Ca_o^{2+}$-elicited decrease in the circulating level of PTH also promotes rapid excretion of calcium by the kidney. This action is thought to take place through a reduction in the PTH-induced stimulation of calcium reabsorption in the thick ascending limb of the loop of Henle as well as the distal convoluted tubule. However, high $Ca_o^{2+}$ also directly inhibits calcium reabsorption by virtue of the presence of the CaR on the basolateral

surface of the epithelial cells of the thick ascending limb, where the receptor antagonizes the stimulatory action of PTH on calcium reabsorption. Both the high $Ca_o^{2+}$-elicited decrease in PTH and the direct inhibitory action of high $Ca_o^{2+}$ on the 1-hydroxylation of 25-hydroxy-vitamin $D_3$ inhibit the production of $1,25(OH)_2$ vitamin $D_3$, thereby reducing the absorption of $Ca_o^{2+}$ by the intestine. All these effects of hypercalcemia on the kidney reduce the level of $Ca_o^{2+}$ toward normal. When combined with the effects of a reduction in PTH and an increase in $Ca_o^{2+}$ on bone, the $Ca_o^{2+}$ homeostatic system provides an elegant mechanism for maintaining near constancy of $Ca_o^{2+}$.

## Nonhomeostatic Functions of the CaR

Although the physiological role of the CaR in cells that do not participate in mineral ion homeostasis still remain to be fully defined, studies of the CaR's functions in these diverse cell types have widened the range of functions that the CaR is known to regulate. These CaR-modulated processes include (1) secretion of peptides, for example, ACTH, gastrin, insulin, growth hormone, and PTHrP; (2) ion channel/transporter activity, for example, aquaporin-2 water channels, nonselective cation channels, voltage-dependent $Ca^{2+}$ channels, and calcium-activated potassium ($K^+$) and other $K^+$ channels; (3) gene expression, for example, of the vitamin D receptor and the CaR; (4) proliferation of colonic and ovarian surface epithelial cells, fibroblasts, and keratinocytes; (5) differentiation of keratinocytes, goblet cells, and mammary epithelial cells; (6) apoptosis of fibroblasts, HEK-293 cells stably transfected with the CaR, and prostate cancer cells; and (7) chemotaxis of preosteoblastic cells and macrophages. Thus $Ca_o^{2+}$ may serve as an extracellular messenger regulating diverse cellular functions, not only in cells directly involved in $Ca_o^{2+}$ homeostasis but also in the variety of additional cell types expressing the CaR that play no apparent role in this process.

## Pharmaceutical Aspects

The CaR has become a drug target as a consequence of its central role in calcium homeostasis and the evidence given earlier that it contributes to hyper- and hypocalcemic states. There are two classes of drugs acting on the CaR. One group activates the receptor; these drugs are called calcimimetics. The other group inhibits the effect of $Ca_o^{2+}$ on the CaR; these agents are called calcilytics.

### Calcimimetics

Calcimimetics are type 2 activators that bind to the receptor's TMDs and potentiate the action of $Ca_o^{2+}$ on the CaR. NPS R-568 and AMG073 are two such agents that shift the curve for $Ca_o^{2+}$-regulated changes in $Ca_i^{2+}$ and PTH release to the left without affecting the maximal or minimal responses (Figure 2). Ongoing clinical trials suggest that calcimimetics may prove to be useful in the treatment of hyperparathyroidism, such as in the secondary hyperparathyroidism that occurs in end-stage renal disease.

### Calcilytics

This group of agents has the opposite effect, inhibiting the receptor and thereby stimulating PTH secretion (Figure 2). This class of drugs could potentially be useful in the treatment of osteoporosis by providing brief pulses of endogenous PTH secretion. The resultant transient increase in PTH mimics that caused by the once-daily administration of exogenous PTH, which is known to exert anabolic actions on bone and has recently been approved for use in the treatment of osteoporosis.

### See Also the Following Articles

Calcium, Biological Fitness of • Mitogen-Activated Protein Kinase Family • Phospholipase $A_2$ • Phospholipase C • Phospholipase D • Protein Kinase C Family

### Glossary

**autosomal dominant hypoparathyroidism (ADH)** A generally benign form of hypocalcemia caused by gain-of-function mutations in the CaR.

**calcilytic** An inhibitor of the CaR.

**calcimetic** The allosteric activator of the CaR.

**calcium sensing receptor (CaR)** A G protein-coupled receptor maintaining the near constancy of extracellular calcium.

**familial hypocalciuric hypercalcemia (FHH)** A benign form of hypercalcemia caused by loss of function in the CaR.

**neonatal severe primary hyperparathyroidism (NSHPT)** An autosomal recessive disease with loss of function of both alleles of the CaR.

### Further Reading

Brown, E. M. (2001). Physiology of calcium homeostasis. In *The Parathyroids* (J. P. Bilezikian, R. Marcus and M. A. Levine, eds.), pp. 167–183. Academic Press, San Diego, CA.

Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, S. C. (1993). Cloning and characterization of an extracellular $Ca^{(2+)}$-sensing receptor from bovine parathyroid. *Nature* **366**, 575–580.

Brown, E. M., and MacLeod, R. J. (2001). Extracellular calcium sensing and extracellular calcium signaling. *Physiol. Rev.* **81**, 239–297.

Tfelt-Hansen, J., Yano, S., Brown, E. M., and Chattopadhyay, N. (2002). The role of the calcium-sensing receptor in human pathophysiology. *Curr. Med. Chem.-Immunol. Endocrinol. Metab. Agents* **2**, 175–193.

## Biography

Jacob Tfelt-Hansen is a Postdoctoral Fellow at Harvard University. His research interests are calcium homeostasis and the non-calcium-homeostatic effects of the CaR. He received his M.D. degree from Copenhagen University, Denmark.

Edward M. Brown is a Professor of Medicine at Harvard Medical School and Brigham and Women's Hospitals. He has worked in the field of calcium homeostasis for nearly 30 years. He received his M.D. degree from Harvard University in 1972. He led the team that cloned the CaR and also played a key role in the identification and characterization of inactivating and activating mutations in the CaR.

# Calcium Signaling: Calmodulin-Dependent Phosphatase

**Claude Klee, Hao Ren and Shipeng Li**
*National Cancer Institute, Bethesda, Maryland, USA*

**Calcineurin (also called protein phosphatase-2B) is a eukaryotic protein phosphatase whose activity is dependent on $Ca^{2+}$ and calmodulin. Ten years ago, the identification of calcineurin as the target of the immunosuppressive drugs, cyclosporin A (CsA) and tacrolimus (FK506), revealed the key role of calcineurin in the transduction pathway from the plasma membrane to the nucleus leading to T cell activation. Since then, the inhibition of calcineurin by FK506 and CsA complexes with their respective binding proteins, FKBP12 and cyclophilin A (CyPA), together with the overexpression of a truncated derivative of calcineurin, not dependent on $Ca^{2+}$ or calmodulin (CaM), have been widely used to probe the involvement of calcineurin in the regulation of cellular processes. These are as diverse as gene expression, ion homeostasis, muscle differentiation, embryogenesis, secretion, and neurological functions. Alteration of calcineurin activity has been implicated in the pathogenesis of many diseases such as cardiac hypertrophy, congenital heart diseases, and immunological and neurological disorders.**

## Subunit Structure and Isoforms

Calcineurin is a heterodimer of a 58–64 kDa catalytic subunit, calcineurin A (CnA), tightly bound to a 19 kDa $Ca^{2+}$-binding regulatory subunit, calcineurin B (CnB). This subunit structure, unique among protein phosphatases, is conserved in all eukaryotes. Three mammalian isoforms ($\alpha$, $\beta$, $\gamma$) of CnA have been identified at the mRNA level. The corresponding human genes (PPP3CA, PPP3CB, PPP3CC) are located on human chromosomes 4, 10, and 8 respectively. Additional isoforms, products of alternative splicing, have only been detected at the mRNA level. Two mammalian isoforms of CnB, CnB1, and CnB2 (expressed only in testis), are the products of two genes (PPP3R1 located on chromosome 2, and PPP3R2). Although expressed in most, if not all tissues, calcineurin is particularly abundant in the brain where the $\alpha$-isoform of CnA is predominant. The $\beta$-isoform is broadly distributed whereas the $\gamma$-isoform is expressed specifically in the testis. Calcineurin has been identified in many other eukaryotes including plants, fungi, and the budding yeast, *Saccharomyces cerevisiae*. Another calmodulin-stimulated protein phosphatase with a CnB-like structure covalently bound to the catalytic subunit has been identified in *Drosophila melanogaster*'s retina.

## Structure

The domain structure of calcineurin is well conserved. In all species the catalytic domain located in the N-terminal two-thirds of CnA, is followed by a CnB-binding domain. The C-terminal regulatory domain is composed of CaM-binding and autoinhibitory subdomains. In the absence of CaM the native protein is inactive. The catalytic and CnB-binding domains, severed from the regulatory domain by limited proteolysis, constitute the $Ca^{2+}$-independent, truncated, form of calcineurin routinely used in overexpression experiments. CnB is an "EF-hand" $Ca^{2+}$-binding protein of the CaM family. It binds four $Ca^{2+}$, two with high affinity ($K_d < 10^{-7}$ M) and two with moderate affinity ($K_d \geq 10^{-6}$M). The N-terminal glycine of CnB is myristoylated. This perfectly conserved posttranslational modification of CnB, apparently not involved in membrane association or required for enzymatic activity, may serve as a stabilizing structural element.

The crystal structure of the recombinant $\alpha$-isoform of human calcineurin as expressed in *E. coli* is illustrated in Figure 1A. The architecture of the catalytic domain is similar to those of protein phosphatases-1 and -2A. It consists of a $\beta$-sandwich surrounded on one side by seven $\alpha$-helices and on the other by a mixed $\alpha/\beta$ structure. The catalytic site contains a $Fe^{3+}$–$Zn^{2+}$ binuclear metal center. The catalytic domain extends into a five-turn amphipathic $\alpha$-helix whose top face is covered by a 33Å groove formed by the N- and C-terminal lobes and the C-terminal strand of CnB. A short helical segment, corresponding to the autoinhibitory domain, blocks the catalytic center. With the exception of this segment, the C-terminal regulatory domain is not visible in the electron density map, and is flexible and highly sensitive to proteolysis, in the absence of CaM. The crystal

 © 2004, Elsevier Inc. All Rights Reserved. 

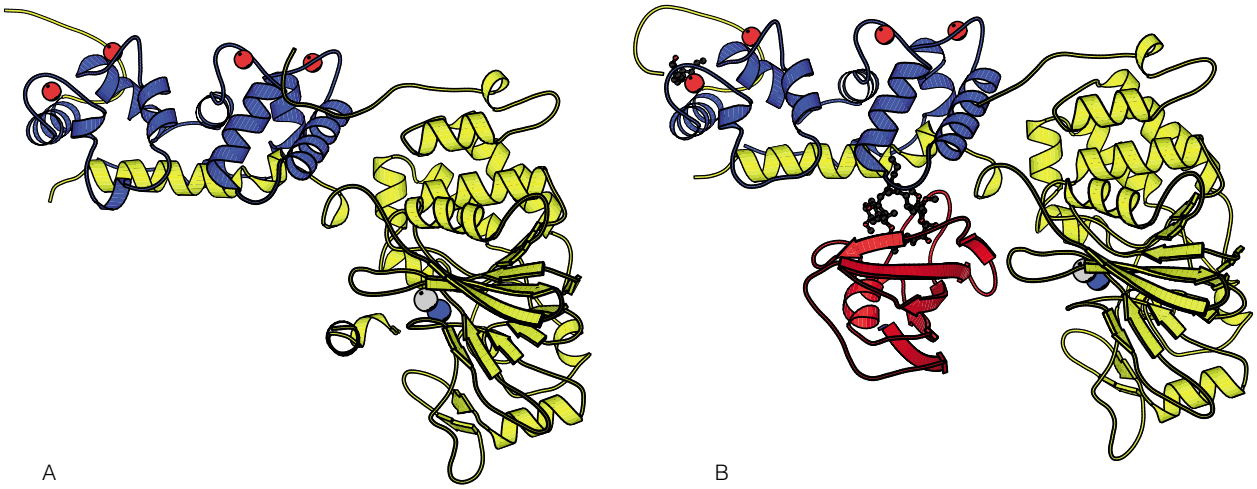



**FIGURE 1** Ribbon representation of the crystal structure of calcineurin. (A) Human recombinant $\alpha$-calcineurin [PDB code 1AUI, Kissinger, C. R., Parge, H. E. *et al.* (1995). *Nature* **378**, 641–644]. (B) Truncated calcineurin complexed with FKBP12-FK506 [PDB code 1TCO Griffith, J. P., Kim, J. L. Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Fleming, M. A., Caron, P. R., Hsiao, K., and Navia, M. A. (1995). X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell* **11**, 507–522. CnA is shown in yellow, CnB in blue. Iron and zinc are shown as light gray and blue spheres respectively. The four calcium bound to CnB are shown as red spheres. FKBP12 is shown in red.

structure of the proteolytic derivative of calcineurin, lacking the regulatory domain, when bound to FK506/FKBP12, is similar to that of the full-length protein (Figure 1B). The bottom face of the CnB-binding helix of CnA and CnB form the site of interaction with the FKBP–FK506 and CsA–CyP complexes. The key role of CnB in forming the drug-binding site provides a molecular basis for the specificity of FK506 and CsA as calcineurin inhibitors.

## Enzymatic Properties

### $Ca^{2+}$/Calmodulin-Dependent Stimulation and Inactivation

Calcineurin is a protein phosphatase with a binuclear iron–zinc active center. The purified enzyme, partially depleted of its metal cofactors, is activated by 0.1 mM $Mn^{2+}$ or 6 mM $Mg^{2+}$. Purified calcineurin, that has retained stoichiometric amounts of $Fe^{3+}$ and $Zn^{2+}$, is inactive and not activated by exogenous metal ions. Activation requires treatment under anaerobic conditions with the reducing agent, ascorbate, which converts $Fe^{3+}$ to $Fe^{2+}$. The phosphatase activity of calcineurin is also dependent on the presence of $Ca^{2+}$ and calmodulin. A small activation is observed upon addition of $Ca^{2+}$ ($K_{act} = 0.5\ \mu M$). Supplementation with CaM results in a 50–100-fold increase of the $V_{max}$ without affecting the value of the $K_m$. The highly cooperative stimulation of the enzyme by CaM (Hill coefficient of 2.5–3) allows calcineurin to respond to narrow $Ca^{2+}$ thresholds following cell stimulation. Because of its high affinity for CaM ($K_{act} < 10^{-10}$ M) the activation of calcineurin in response to a $Ca^{2+}$ signal can precede the activation of all known CaM-regulated kinases.

### Enzymatic Assays

A 19-residue synthetic peptide containing the phosphorylation site of the RII subunit of cAMP-dependent protein kinase (PKA) and *p*-nitrophenylphosphate are used routinely to measure the phosphatase activity of calcineurin. In crude tissue extracts calcineurin activity is distinguished from that of other neutral serine–threonine protein phosphatases by its (1) $Ca^{2+}$ dependence; (2) inhibition by CaM-binding peptides and inhibitors; (3) resistance to the inhibitors of protein phosphatases-1 and -2A (Inhibitor-1, DARPP32, Inhibitor-2, okadaic acid, microcystin, and calyculin); and (4) specific inhibition by FK506 (but not rapamycin) and CsA in the presence of saturating amounts of their respective binding proteins, FKBP12 and CyPA. The *p*-nitrophosphatase activity of calcineurin, which is stimulated by FK506 and CsA, cannot be used to measure calcineurin activity in crude extracts. Unlike protein phosphatase-2C, which requires $Mg^{2+}$ for activity, calcineurin that has retained its natural metal cofactors does not require $Mg^{2+}$.

### Substrate Specificity

Calcineurin is a dual protein phosphatase. It dephosphorylates both phosphoseryl/threonyl and phosphotyrosyl peptides. Peptides with a basic residue on the

N-terminal side of the phosphorylated amino acid and lacking acidic residues on the C-terminal side are preferentially dephosphorylated by calcineurin. The substrate specificity of calcineurin also depends on an extended N-terminal stretch. In addition to the recognition of the sequence surrounding the phosphorylated residues, the presence of docking domains greatly decrease the $K_m$ values. A major substrate of calcineurin, the transcription factor NFAT (Nuclear Factor of Activated T cells), contains two such domains responsible for its $Ca^{2+}$-dependent and phosphorylation-independent anchoring to calcineurin. The anchoring of NFAT allows its dephosphorylation, despite its low intracellular concentration, and the nuclear cotranslocation of calcineurin and NFAT.

## Regulation

### $Ca^{2+}$ Regulation

Two structurally similar but functionally different $Ca^{2+}$-binding proteins, CaM and CnB mediate the $Ca^{2+}$ regulation of calcineurin. CnB is tightly bound to CnA even in the absence of $Ca^{2+}$, and plays both structural and functional roles. It is required to confer enzymatic activity to the catalytic subunit. The recombinant $\alpha$- and $\beta$-isoforms of CnA expressed in bacteria and SF9 cells require CnB for activity. $Ca^{2+}$-binding to CnB is required for the interaction of calcineurin with immunosuppressive drugs bound to their binding proteins.

$Ca^{2+}$-dependent binding of CaM to calcineurin induces a conformational change of the regulatory domain accompanied by the displacement of the autoinhibitoy domain. The resulting exposure of the catalytic center allows substrate binding and results in activation of the phosphatase activity. Prolonged exposure of the catalytic center facilitates the oxidation of $Fe^{2+}$ and results in inactivation of calcineurin that is prevented by superoxide dismutase and reversed by ascorbate. Thus, CaM can both activate and inactivate calcineurin depending of the length of the $Ca^{2+}$ signal. The calmodulin-dependent oxidation of $Fe^{2+}$ and inactivation of calcineurin is a potential mechanism to regulate its activity during oxidative stress.

### Endogenous Regulatory Proteins

Endogenous protein inhibitors or activators, whose expression varies from tissue to tissue, can also modulate the activity of calcineurin activity *in vivo*. A family of 22–24 kDa proteins, identified in both yeast (RCn1p) and mammalian cells (calsuppressins, MCPs), act as feedback inhibitors of calcineurin. The mammalian proteins, whose expression is up-regulated by a calcineurin/NFAT-dependent mechanism, are identical to the proteins encoded in the Down's syndrome Critical Region 1 gene, ZAK1–4, DSCRIL1, and DSCRIL. These inhibitory proteins inhibit both calcineurin expression and activity. They interact with calcineurin in the absence of $Ca^{2+}$ through a highly conserved ISPP × SPP motif, similar to the SP motifs of NFAT, and inhibit calcineurin activity *in vitro* as well as NFAT activation *in vivo*.

A 240 kDa nuclear protein, Cain/Cabin1 has been identified as a non-competitive inhibitor of calcineurin. Binding of Cabin1 to calcineurin requires $Ca^{2+}$ and protein kinase C activation and is inhibited by FK506/FKBP, suggesting that it binds at the drug interaction site. A basic domain in the C terminus of Cabin1 has been identified as the calcineurin-binding site. A third group of proteins, including the PKA scaffold protein, AKAP79 in brain, and calsarcins in skeletal and cardiac muscles may serve to anchor calcineurin to its sites of action. They all inhibit calcineurin activity but their mechanism of action is not yet fully understood.

## Functions

### Regulation of Gene Expression

The elucidation of the $Ca^{2+}$/calcineurin/NFAT pathway of T cell activation opened the way to uncover the important role of calcineurin in the regulation of gene expression in many other cell types. As illustrated on Figure 2, in T cells, when $Ca^{2+}$ is released from the inositol trisphosphate ($IP_3$)-sensitive stores following occupancy of the T cell receptor, calcineurin is activated and dephosphorylates the transcription factor NFAT. Dephosphorylation of NFAT is accompanied by the exposure of a nuclear localization signal and NFAT is translocated to the nucleus. Calcineurin, anchored to NFAT through an anchoring domain (P × I × IT), is translocated to the nucleus as well. Dephosphorylation of NFAT not only results in its nuclear translocation but also in an enhancement of its DNA binding and transcriptional activity. This is also dependent on the activation of the Ras/protein kinase C (PKC) pathway. Nuclear export depends on NFAT rephosphorylation upon removal of $Ca^{2+}$ and calcineurin inactivation. Several kinases, including glycogen synthase kinase 3 (GSK3) and casein kinase 1 have been implicated in this process.

Four NFAT isoforms (NFAT1–4) are expressed in many tissues. These isoforms share a highly conserved regulatory domain composed of two calcineurin-binding motifs, multiple phosphorylation sites, and a nuclear localization signal. The two motifs bind calcineurin with an affinity in the micro molar range. In skeletal

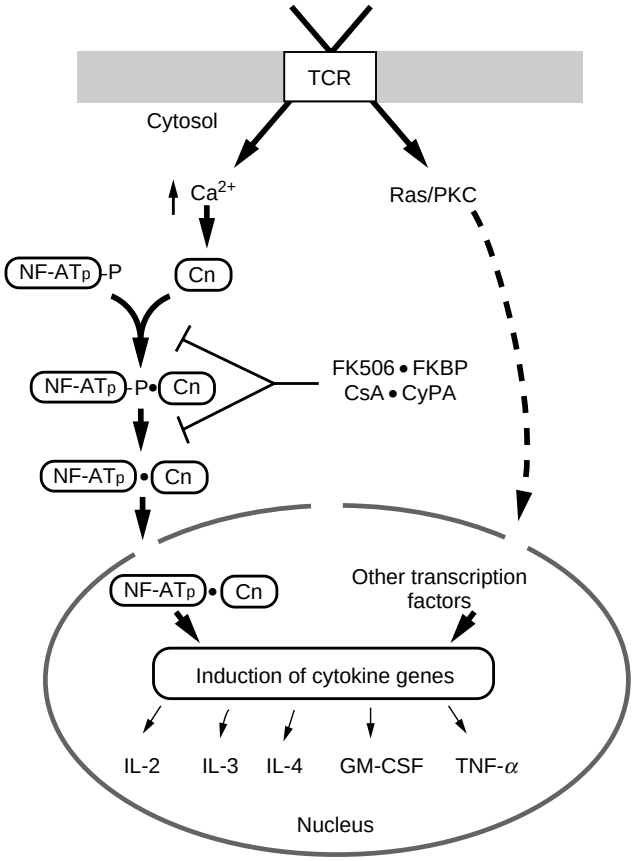



**FIGURE 2** Calcineurin-mediated signal transduction from the T cell receptor to the nucleus leading to the induction of the cytokine genes (IL-2, -3, -4), granulocyte-macrophage stimulating factor (GM-CFS), and tumor necrosis factor (TNF-$\alpha$). Gene activation requires the concomitant activation of the Ras/protein kinase C (PKC) pathway. [Reproduced from Klee, C. B., Wang, X., and Ren, H. (1999). Calcium-regulated protein dephosphorylation. In *Calcium as a Cellular Regulator* (Carafoli and Klee, eds.) with permission of Oxford University Press, Inc.]

muscle calcineurin-mediated dephosphorylation of the transcription factors, NFAT-3 and the myogenic enhancing factor (MEF2) plays a critical role in the switch from fast-to-slow oxidative fibers induced by sustained tonic contraction. In the heart, the calcineurin-mediated activation of NFAT3 and MEF2D, in conjunction with the cardiac specific transcription factor GATA4, has been implicated in the development of cardiac hypertrophy in response to stress. Transgenic mice lacking the $\beta$-isoform of calcineurin (the predominant isoform in heart) have impaired ability to develop cardiac hypertrophy in response to hypertrophic agonists while severe hypertrophy and cardiac heart failure is induced by overexpression of constitutively active calcineurin. The calcineurin/NFAT pathway is also essential for the development of heart valves and the vascular developmental pattern during embryogenesis. In the brain, $Ca^{2+}$ homeostasis is itself regulated by calcineurin. Activation of NFAT increases the expression of the $IP_3$ receptors, the plasma membrane $Ca^{2+}$ pumps and the $Ca^{2+}$ exchanger. The increasing number of genes whose expression is induced by NFAT suggests a potential role of calcineurin in the regulation of gene expression during cell differentiation and apoptosis. The importance of calcineurin in the regulation of these cellular processes and its involvement in the pathogenesis of many diseases based on overexpression and knock out models is well documented. To fully assess the contribution of calcineurin in the transduction of so many diverse signals, we must understand how different pathways interact with each other.

In budding yeast, the calcineurin-mediated dephosphorylation of a transcription factor (Crz1p/Tcn1p) plays a major role in the regulation of genes involved in ion homeostasis. The mechanism is similar to NFAT-mediated transcriptional control in mammalian cells. In yeast, genetic analysis adds strong support to the physiological significance of this calcineurin signaling pathway in response to exposure to abnormal concentrations of ions or prolonged exposure to mating pheromones. It not only illustrates the important role of calcineurin in the regulation of gene expression but also reveals the participation of many other factors in this complex signaling pathway.

## Neuronal Functions

Calcineurin is 1% of the total protein and broadly distributed in brain. There are few neuronal functions which are not modulated by calcineurin. A major role of calcineurin in brain is to trigger a protein phosphatase cascade initiated by the dephosphorylation of two other major substrates of calcineurin, the endogenous inhibitors of protein phosphatase-1, Inhibitor-1, and DARPP32. This cascade counteracts the stimulatory effects of cAMP and $Ca^{2+}$-regulated kinases. It explains the antagonistic effects of $Ca^{2+}$ release induced by glutamate binding to the NMDA receptor and dopamine binding to some dopamine receptors in striatal neurons. Whether it activates protein phosphatase-1, or dephosphorytes an increasing number of specific substrates, calcineurin has been implicated in the regulation of neuronal processes as diverse as the expression and activity of ion channels, the synthesis of nitric oxide, the release of neurotransmitters, synaptic vesicle recycling, and neurite outgrowth. Calcineurin inhibitors and transgenic animals lacking the calcineurin genes or overexpressing the constitutive form of calcineurin have been widely used to study the involvement of calcineurin in the storage of long and short memory. How calcineurin and other protein kinases and phosphatases affect learning and memory remains one of the most challenging problems in neurobiology.

## See Also the Following Articles

Calcium/Calmodulin-Dependent Protein Kinase II • Protein Kinase C Family

## Glossary

**calmodulin** A unique member of a class of calcium-binding proteins that acts as sensors of changes in intracellular concentration of calcium induced by external signals. It modulates the activity of a large number of enzymes involved in the regulation of cellular processes, particularly those involved in protein phosphorylation.

**EF-hand** Structural motif composed of 30 contiguous amino acids forming two helices joined by a $Ca^{2+}$-binding loop.

**immunosuppressive drugs (FK506 and CsA)** Fungal natural products used to prevent organ rejection after organ transplant operations and treatment of autoimmune diseases.

**NFAT** Broadly distributed transcription factor originally identified as a nuclear factor of activated T cells required for the coordinated induction of several cytokine genes.

## Further Reading


Aramburu, J., Rao, A., and Klee, C. B. (2000). Calcineurin: From structure to function. *Curr. Top. Cell. Regul.* **36**, 237–295.

Barford, D., Da, A. K., and Egloff, M.-P. (1998). The structure and mechanism of protein phosphatases: Insights into catalysis and regulation. *Annu. Rev. Biophys. Biomol. Struct.* **27**, 133–164.

Bito, H., Deisseroth, K., and Tsien, R. W. (1996). CREB phosphorylation and dephosphorylation: A $Ca^{2+}$-and stimulus duration-dependent switch for hippocampal gene expression. *Cell* **87**, 1203–1214.

Cyert, M. S. (2001). Genetic analysis of calmodulin and its targets in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **35**, 647–672.

Fienberg, A. A., Hiroi, N., Mennelstein, P. G., Song, W., Snyder, G. L., Nishi, A., Cheramy, A., O'Callaghan, J. P., Miller, D. B., Cole, D. G., Corbett, R., Haile, C. N., Cooper, D. C., Onn, S. P., Grace, A. A., Ouimet, C. C., White, F. J., Hyman, S. E., Surmeier, D. J., Girault, J., Nestler, E. J., and Greengard, P. (1998). DARPP-32: Regulator of the efficacy of dopaminergic neurotransmission. *Science* **281**, 838–842.

Graef, I. A., Chen, F., Chen, L., Kuo, A., and Crabtree, G. (2001). Signals transduced by $Ca^{2+}$/calcineurin and N-F-AT c3/c4 pattern the developing vasculature. *Cell* **105**, 863–875.

Griffith, J. P., Kim, J. L. Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Fleming, M. A., Caron, P. R., Hsiao, K., and Navia, M. A. (1995). X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell* **11**, 507–522.

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**, 1030–1038.

Kissinger, C. R., Parge, H. E. *et al.* (1995). *Nature* **378**, 641–644.

Klee, C. B., Wang, X., and Ren, H. (1999). Calcium-regulated protein dephosphorylation. In *Calcium as a Cellular Regulator.* (C. Carafoli, C. Klee, eds.) pp. 344–370. Oxford University Press, New York.

Malleret, G., Haditsch, U., Genoux, D., Jones, M. W., Bliss, T. V., Vanhoose, A. M., Weitlauf, C., Kandel, E. R., Winder, D. G., and Mansuy, I. M. (2001). Reversible enhancement of leaming, memory, and long-term potentiation by genetic inhibition of calcineurin. *Cell* **104**, 675–686.

McKinsey, T. A., Zhang, C. L., and Olson, R. N. (2002). Signaling chromatin to make muscle. *Curr. Opin. Cell Biol.* **14**, 763–772.

Schreiber, S. L., Liu, J. L., Albers, M. W., Rosen, M. K., Standaert, R. F., Wandless, T. J., and Somer, P. K. (1992). Molecular recognition of immunophilins and immunophilinligand complexes. *Tetrahydron* **48**, 2545–2558.

Winder, D. G., and Sweatt, J. D. (2001). Roles of serine/threonine phosphataes in hippocampal synaptic plasticity. *Nat. Rev. Neurosci.* **2**, 41–474.


## Biography

Claude B. Klee is Scientist Emeritus in the Laboratory of Biochemistry at the National Cancer Institute in Bethesda, MD. Her principal research interest is in $Ca^{2+}$ signaling with emphasis on the mechanism of action of calmodulin using as a model system the activation of the calmodulin-dependent protein phosphatase, calcineurin. She holds an M.D. from the University of Marseille, France, and is a member of the Institute of Medicine and the American Association of Arts and Sciences.

Hao Ren and Shipeng Li are long time associates of Dr. Klee. They developed in her laboratory the procedures to express human calcineurin in bacteria, and to characterize the enzymatic activity and calcium regulation of the recombinant proteins.

# Calcium Signaling: Cell Cycle

**Luigia Santella**
*Stazione Zoologica "Anton Dohrn," Naples, Italy*

**Because calcium controls the most important cellular functions, it is not surprising that it should also have a role in a process as vital as the ability of cells to divide and multiply. Eukaryotes from yeasts to humans have similar division cycles, which are carefully regulated and coordinated with both cell growth and DNA replication to ensure the formation of a progeny of cells containing intact genomes. This is made possible by the existence of a system of checkpoints and feedback controls that prevent the entry of cells into the next phase of the cycle if the preceding phase has not been successfully completed.**

**A large number of regulatory elements have a function in the various stages of the cell cycle, among them, a variety of kinases, phosphatases, proteases, and a number of second messengers that control them. Among second messengers that have a role in the regulation of the cycle, calcium is a relatively late entry, but is now attracting increasing attention. This short article will offer a succinct survey of its role, paying attention to the meiotic cycle as well. For more detailed information, readers may consult a number of comprehensive reviews that deal with the general topic of cell-cycle regulation.**

## The Mitotic Cycle

The cell division is conventionally subdivided into four stages: $G_1$, S, $G_2$, and M. The M phase usually ends with the formation of two daughter cells (cytokinesis) (Figure 1). The $G_1$ phase corresponds to the interval gap that starts at the completion of mitosis and extends to the initiation of DNA synthesis. During this phase, the cell grows continuously and synthesizes the histones necessary for the new chromatin. $G_1$ is also the phase in which the cell responds to external signals by becoming committed irreversibly to enter the replicative process. This occurs at a point of no return called the restriction point (R) (in yeast it is named start). When proliferating cells are deprived of the appropriate medium they exit from the cycle after having reached the $G_1$ phase and enter a quiescent state termed $G_0$ in which they stop growing. DNA replication occurs in the S phase. The $G_2$ phase is the time-gap between the completion of DNA synthesis and the beginning of chromosome condensation. During $G_2$ phase, mRNAs and proteins are synthesized in preparation for the M phase (M), which consists of four steps: prophase, metaphase, anaphase, and telophase. The beginning of prophase is marked by the appearance of condensed chromosomes with the two sister chromatids. During prophase, in addition to chromosome condensation, changes leading to the development of the mitotic spindle also occur in the cytoplasm. The centrosomes, which had duplicated during interphase and which serve as the two poles of the mitotic spindle, separate and move to opposite sides of the nucleus. The breakdown of the nuclear envelope then allows spindle microtubules to attach to the kinetochores of chromosomes. At the metaphase stage the chromosomes align in the center of the spindle, and the sister chromatids separate, to move to opposite poles of the spindle during the anaphase stage. Chromosomes decondensation and nuclear envelope reformation take place during telophase, after which cytokinesis yields two interphase daughter cells.

### Cell-Cycle Regulators

The central actor in the initiation and progression of the cell cycle is a well-conserved family of serine/threonine protein kinases (CDKs), which become activated by associating with cyclins. This binding is strictly necessary for CDKs activity and cyclin protein levels are tightly controlled during the cell cycle. Various cyclins become synthesized and degraded at specific phases of the cycle. In mammalian cells, three cyclins (C, D1, and E) that are essential for entering S phase are synthesized during the $G_1$ phase. The D-cyclins, which control the restriction point R, form complexes with CDK4 and CDK6, and are regulated by external signals, such as growth factors, anti-mitogenic factors, etc. Cyclin E is synthesized late in $G_1$ and once the active complex CDK2/cyclin E is formed the active kinase triggers the onset of DNA replication. Cyclin A and B, that are essential for M-phase entry, are synthesized during $G_2$. At the end of M phase, the ubiquitin-dependent proteasome pathway, which is required to inactivate CDK1 and to allow cells to enter $G_1$, degrades mitotic cyclins. However, it has been claimed that one of the cylins (D1) is processed by calpain.

 © 2004, Elsevier Inc. All Rights Reserved. 

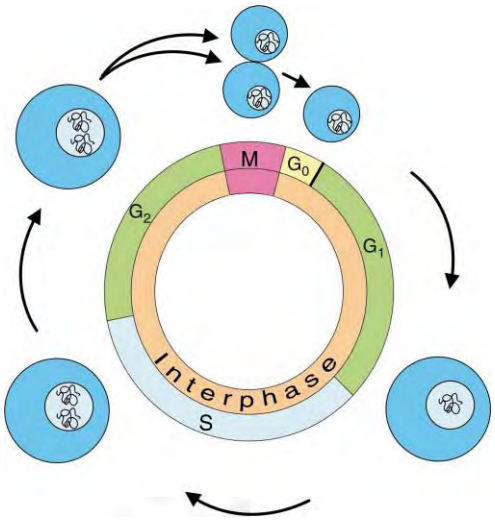



**FIGURE 1** Cell-cycle phases. The cell grows throughout interphase, which includes $G_1$, S, and $G_2$. Once the cell has passed through the restriction point, it is committed to proceed through S and the rest of the cycle. If growth factors are not available during $G_1$, the cell stops growing and protein synthesis becomes depressed. S phase is the period during which DNA replication occurs. During $G_2$ mRNAs and proteins are synthesized in preparation for the mitosis (M) that ends with the two daughter cells.

### Calcium and Mitosis

Although the mechanisms responsible for the activation of the cell-cycle kinases are still incompletely understood, increasing evidence now strongly suggest that changes in intracellular calcium may have an important role. During the early embryonic cell cycle in the sea urchin, cyclic increases in inositol 1,4,5-trisphosphate trigger the release of $Ca^{2+}$ from intracellular stores that generate mitotic events. Blocking of the calcium increase prevents the dissolution of the nuclear envelope and stops mitosis. Calcium transients are associated with chromosome movement during anaphase in plants and mammalian cells. Calcium and the principal decoder of its signal, the calcium-binding protein, calmodulin (CaM), are intimately connected to the events of the cycle. Anti-CaM drugs block the $G_1$–S transition and the onset of DNA replication. The CaM level increases as the cell progress into mitosis, correlating nicely with the detection of CaM in the mitotic apparatus in yeast. Local activation of CaM occurs near the nuclear envelope just before its breakdown and the onset of anaphase. CaM is also involved in the phosphorylation of the retinoblastoma protein, the product of the Rb1 tumor suppressor gene (which is responsible for a rare inherited childhood eye tumor) that modulates a set of regulatory proteins that promote cell proliferation. The retinoblastoma protein is one of the key regulators that control R in $G_1$, past which the cells become irreversibly committed to enter S phase. The multifunctional $Ca^{2+}$/CaM-dependent protein kinase II ($Ca^{2+}$/CaMKII) is a potential transducer of the effect of CaM on cell-cycle progression. Inhibition of the $G_2$/M transition, and thus of the nuclear envelope breakdown, occurs upon injection of a $Ca^{2+}$/CaMKII specific peptide inhibitor or of $Ca^{2+}$/CaMKII antibodies. The involvement of $Ca^{2+}$/CaMKII at mitosis and meiosis entry is due to the activation of a Cdc25, a phosphatase that is a $Ca^{2+}$/CaMKII substrate that dephosphorylates and which activates, the M-phase promoting factor (MPF). The kinase induces multiple nuclear and cytoplasmic changes at the onset of the M phase by activating other protein kinases, and by phosphorylating structural proteins such as histone H1, nuclear lamins, proteins of the endoplasmic reticulum (ER) and Golgi membranes, and microtubule and actin-associated proteins. Once the metaphase spindle has been formed the cell proceeds to initiate anaphase and complete meiosis. Progression from metaphase to anaphase is triggered by the activation of an ubiquitin-mediated proteolysis system (proteasome) that degrades cyclin B and thereby inactivates MPF. In turn, phosphorylation of the proteasome by activated $Ca^{2+}$/CaMKII promotes cyclin degradation. As mentioned $Ca^{2+}$ may also intervene in the degradation of cyclins by activating the $Ca^{2+}$-dependent protease calpain, that has been shown to degrade cyclin D1.

## The Meiotic Cycle

### Oocyte Prophase and Metaphase Arrest

Oocytes have long been used to study the meiotic cycle. In the gonads their growth is arrested at a specific phase of the meiotic cycle (prophase I). Following growth, a hormonal signal causes oocytes to mature. During maturation oocytes undergo a number of morphological, electrical, and biochemical changes that are a necessary preparation for successful fertilization. At the onset of maturation, the envelope of the germinal vesicle (nucleus) of prophase I-arrested oocytes disassembles (GVBD), the nucleoplasm mixes with the cytoplasm and the ER undergoes a structural reorganization that will ensure a normal $Ca^{2+}$ response at fertilization: data indicate that MPF plays an important role in the restructurating of the ER. In addition to the dissolution of the nuclear envelope, resumption of meiosis entails the condensation of chromatin and the extrusion of the first polar body. The latter results from an unequal cell division that leaves most of the cytoplasm with half of the DNA. Following the release of the first polar body, oocytes enter the second mitosis of the meiotic cycle with the

extrusion of the second polar body and the formation of the pronucleus.

Although the pathway leading to the release from prophase I differs significantly among species, its ultimate common goal is to activate MPF. The experiments that have contributed to the identification of MPF as the agent responsible for the mitotic and meiotic cycle regulation have been performed on frog and starfish oocytes. Arrested oocytes could be induced to enter the M phase of meiosis by microinjecting cytoplasm from oocytes that had been hormonally stimulated, i.e., the cytoplasmic factor generated in hormone-treated oocytes was evidently sufficient to trigger the transition from $G_2$ to M in oocytes that had not been exposed to the hormone.

## Calcium and Meiosis

Abundant evidence links the meiotic division cycle to calcium. For example, prophase I arrested starfish oocytes release calcium in the cytoplasm and in the nucleus following the resumption of meiosis promoted by the maturation-inducing hormone 1-methyladenine. A role of calcium during maturation is indicated by the inhibition of meiosis progression by the injection of CaM antagonists or of CaM antibodies directly into the oocytes nucleus. MPF activation could thus occur in the nuclear compartment, and could be linked to the $Ca^{2+}$/CaMKII-dependent stimulation of the Cdc25 phosphatase, which dephosphorylates two inhibitory (Th-14 and Tyr-15) MPF sites. In line with this, both MPF and Cdc25 have been shown to migrate to the nucleus once the re-initiation of meiosis has started. In some species in which fertilization triggers the release from prophase I, oocytes complete meiosis and enter mitosis directly without arrest. However, in most species in which hormonal stimuli rather than fertilization trigger the release from prophase I, the oocytes become arrested again at either metaphase I, metaphase II, or the pronucleus stage (depending on the species), until fertilization (Figure 2). For instance, *Xenopus* oocytes become arrested for a second time after maturation at the metaphase of the second meiotic division: The cytoplasmic activity that causes metaphase II arrest is termed the cytostatic factor. Injection of cytoplasm from metaphase-arrested oocytes that contains the cytostatic factor into a dividing blastomere results in the arrest of the recipient at metaphase. A serine/threonine kinase termed Mos was first identified as an essential component of the cytostatic factor. Mos is specifically synthesized in oocytes at about the time of completion of meiosis I and is responsible for the maintenance of MPF activity during the metaphase II arrest. Mos can readily arrest a dividing blastomere, and its

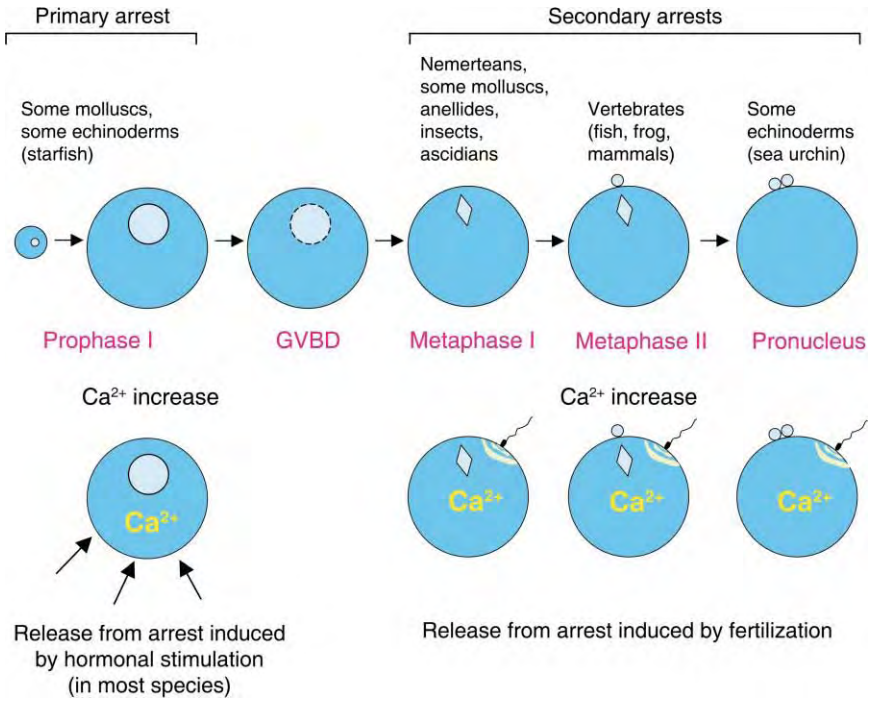



**FIGURE 2** Release from meiotic arrest in different animal oocytes. In most species prophase I-arrested oocytes at the germinal vesicle (nucleus) stage (GV) re-initiate the meiotic cycle following the intracellular $Ca^{2+}$ increase induced by hormonal stimulation. In a minority of species, however, fertilization may induce the release from prophase I arrest in the absence of hormonal stimulation. Then, in the species in which the release is induced by the hormone, a secondary arrest may occur (however, not in starfish oocytes) at metaphase I and II that is released by the intracellular $Ca^{2+}$ increase promoted by sperm entry at fertilization.

immunodepletion removes cytostatic factor activity. The release of the secondary arrest is promoted by the entry of the sperm at fertilization and by the associated $Ca^{2+}$ wave produced by the emptying of the intracellular stores. The $Ca^{2+}$ increase inactivates both the cytostatic factor and the MPF, inactivation of MPF occurring through the ubiquitin-dependent proteolysis of the regulatory cyclin B. At variance with the oocytes in which the entry of the sperm induces a single $Ca^{2+}$ transient (a $Ca^{2+}$ wave), e.g., those of *Xenopus*, sea urchin, and starfish, in other oocytes species fertilization triggers, instead, repetitive $Ca^{2+}$ transients. This occurs in nemertean, ascidian, and mammalian oocytes, which become fertilized at metaphase I and II when MPF activity is very high. In these species, $Ca^{2+}$ fails to inactivate MPF, whose activity remains high and might actually regulate the $Ca^{2+}$ oscillations induced by the sperm.

## See Also the Following Articles

Cell Cycle: Mitotic Checkpoint • Homologous Recombination in Meiosis • Meiosis • Metaphase Chromosome • Mitosis • The Neuronal Calcium Signal in Activity-Dependent Transcription

## Glossary

**endoplasmic reticulum (ER)** A cytoplasmic network of membrane-enclosed tubules and cisternae extending from the nuclear envelope through the cytoplasm that regulates the intracellular $Ca^{2+}$ levels.

**germinal vesicle** The large nucleus of fully grown, prophase I-arrested oocytes.

**inositol 1,4,5-trisphosphate** An intracellular second messenger produced by the hydrolysis of membrane phospholipid through the activation of a specific phospholipase following interaction of first messengers with specific plasma membrane receptors.

**M-phase promoting factor (MPF)** A cyclin-dependent kinase complex formed by the catalytic subunit of the protein kinase CDK1, and a regulatory subunit, cyclin B.

## Further Reading

Carafoli, E., Santella, L., Branca, D., and Brini, M. (2001). Generation, control, and processing of cellular calcium signals. *Crit. Rev. Biochem. Mol. Biol.* **36**, 107–260.

Carroll, J. (2001). The initiation and regulation of $Ca^{2+}$ signalling at fertilization in mammals. *Semin. Cell Dev. Biol.* **12**, 37–43.

Means, A. R. (1994). Calcium, calmodulin and cell cycle regulation. *FEBS Lett.* **347**, 1–4.

Runft, L. L., Jaffe, L. A., and Mehlmann, L. (2002). Egg activation at fertilization: Where it all begins? *Dev. Biol.* **245**, 237–254.

Santella, L. (1998). The role of calcium in the cell cycle: Facts and hypotheses. *Biochem. Biophys. Res. Commun.* **244**, 317–324.

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* **211**, 157–176.

Whitaker, M., and Patel, R. (1990). Calcium and cell cycle control. *Development* **108**, 525–542.

## Biography

Luigia Santella is a Senior Scientist in the Laboratory of Cell Biology of the Stazione Zoologica "A. Dohrn", Naples, Italy. Her principal research interest is the spatio-temporal dynamics of $Ca^{2+}$ signaling during the cell cycle and fertilization in invertebrate marine animals. She has also contributed to the clarification of the role of the actin cytoskeleton in modulating the intracellular $Ca^{2+}$ increase.

# Calcium Signaling: Motility (Actomyosin–Troponin System)

**Takeyuki Wakabayashi**
*Teikyo University, Utsunomiya, Japan*

**Setsuro Ebashi**
*National Institute for Physiological Sciences, Myodaiji, Japan*

**Muscle contraction is regulated by the level of intracellular $Ca^{2+}$. In skeletal or cardiac muscles of vertebrate, $Ca^{2+}$ regulation is mediated at the level of thin filaments, which consist of troponin, tropomyosin, and actin. Because actin filaments themselves are $Ca^{2+}$-insensitive, troponin and tropomyosin, which form a $Ca^{2+}$-sensitive switch, are required. When the cytosolic $Ca^{2+}$ concentration increases to a micromolar level, $Ca^{2+}$ binds to troponin and triggers a series of conformational changes in the thin filaments that lead to a sliding interaction between actin-containing thin filaments and myosin-containing thick filaments.**

## Structure of Thin Filaments and their Components

Thin filaments are based on a helical arrangement of actin monomers (F-actin), which can be thought of as two strands of globular actin monomers (G-actin) twisted around one another, staggered by ~27.3Å (2.73 nm) (Figure 1). One crossover (half pitch) containing approximately 13 monomers is ~360Å in length. Actin can be divided into the outer domain (subdomains 1 and 2) and the inner domain (subdomains 3 and 4) (Figure 1B). The inner domain is nearer to the helix axis. Myosin binds mainly to the subdomain 1.

In the generally accepted model of the thin filament, tropomyosin molecules are located in the two grooves of actin. Troponin binds to each of tropomyosin molecules as shown schematically in Figure 1A. The native thin filaments also contain nebulin molecules (not shown in Figure 1). One tropomyosin–troponin unit corresponds to exactly seven actin monomers, and the troponin molecules on the opposite strands are staggered with respect to one another by 27.3Å. This had been assumed for the sake of simplicity, but was recently shown to actually be the case. Tropomyosin is a rod-shaped coiled-coil dimer (~400Å, $M_r$ ~35,000 × 2). Tropomyosin molecules form continuous ropes by longitudinal association through head-to-tail contact. Troponin consists of three subunits: troponin-T (TnT, $M_r$~31,000) binds to tropomyosin, troponin-I (TnI, $M_r$~21,000) binds to actin and inhibits the actin-activated myosin ATPase, and troponin-C (TnC, $M_r$ ~ 18,000) binds calcium ions and neutralizes the inhibitory activity of TnI. Troponin, therefore, can bind to both tropomyosin and actin (Figure 2). Troponin can be dissociated into its three subunits in the presence of urea and $Ca^{2+}$-chelating reagents and can be reassembled by removing urea.

### Tropomyosin Shift and Steric Blocking Model

The thin filament model shown in Figure 1A suggests that $Ca^{2+}$-induced changes of troponin can be transmitted to actin monomers through tropomyosin. The simplest hypothesis in calcium regulation has been the steric blocking model, in which the azimuthal position of tropomyosin on actin filaments is considered to be critical.

The increase in the second-actin-layer line intensity of the X-ray diffraction pattern from living muscle and the concomitant decrease in the third-actin-layer line intensity take place during the activation of muscle contraction. These changes in X-ray intensity can be explained if tropomyosins are located near the grooves of the actin double strands in the contracting state but dislocated more from the grooves due to the influence of $Ca^{2+}$-deprived troponin. If the tropomyosins in the off-groove position are located where they block sterically the myosin-binding sites of actin but the tropomyosins in the in-groove position do not, the tropomyosin shift can explain why tropomyosin is required for $Ca^{2+}$-regulation: Tropomyosin transmits the $Ca^{2+}$-induced changes in one troponin to the seven actin monomers. Three-dimensional image reconstruction from electron micrographs showed more directly the changes in thin filament

 © 2004, Elsevier Inc. All Rights Reserved. 

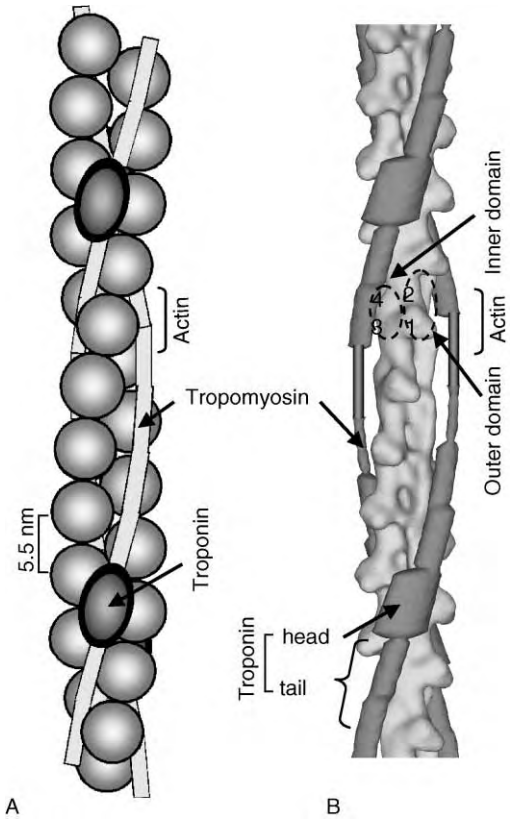



**FIGURE 1** (A) Schematic illustration of a thin filament based on the model shown in (B). One tropomyosin molecule binds seven actin monomers and one troponin. The model in (B) is a composite illustration – the actin part is based on the atomic model of actin, and the troponin and tropomyosin part is based on the three-dimensionally reconstituted image of the actin–tropomyosin–troponin complex (reconstituted thin filament). Smoothing along the actin helix was applied to the tropomyosin and troponin part to simplify it. The four subdomains of actin are indicated by numerals, and the approximate location of the head and tail domains of troponin are indicated. Tropomyosin is ~400Å in length. Reprinted from Narita, A., Yasunaga, T., Ishikawa, T., Mayanagi, K., and Wakabayashi, T. (2001). $Ca^{2+}$-induced switching of troponin and tropomyosin on actin filaments as revealed by electron cryo-microscopy. *J. Mol. Biol.* **308**, 241–261, copyright 2001, with permission from Elsevier.

structure – the addition of TnT–TnI complex (which inhibits actin–myosin interaction irrespective of $Ca^{2+}$ concentration) to the actin–tropomyosin complex induces the tropomyosin shift. It was later shown that the three-dimensional images of reconstituted thin filaments containing actin, tropomyosin, and troponin were changed by the addition of $Ca^{2+}$, and the structural changes were interpreted to be the result of the tropomyosin shift induced by $Ca^{2+}$. The addition of TnI–TnC complex (without TnT) to the actin–tropomyosin complex also induces the tropomyosin shift in a $Ca^{2+}$-dependent manner. There are indications that troponin movement and conformational changes of actin are also involved in $Ca^{2+}$ regulation, and the changes in the X-ray diffraction pattern therefore could be explained solely by the shift of troponin instead of tropomyosin. The positions of tropomyosin determined by modeling or helical image reconstruction are therefore not unambiguous and are biased by changes in the other components of thin filament, especially troponin, as described next.

### Three-State Model for $Ca^{2+}$ Regulation

Biochemical studies showed that the simple steric blocking model, which proposes that only the position of tropomyosin is responsible for $Ca^{2+}$ regulation, is an oversimplification. The binding of the myosin head (subfragment-1, S1) to the reconstituted thin filaments (actin–tropomyosin–troponin complex) is cooperative irrespective of $Ca^{2+}$ concentration and the $Ca^{2+}$ binding to troponin alone is not sufficient for the full activation of actin-activated myosin ATPase.

The cooperative/allosteric model, or three-state model, emphasizes the importance of conformational changes in actin. At low $Ca^{2+}$ concentrations, the thin filament is in a blocked state, in which myosin cannot bind to actin. There are two states at high $Ca^{2+}$ concentrations: an open state (called R state in the allosteric model), in which myosin bound with ADP-phosphate can interact with actin weakly and the weak binding can be converted to a strong binding by releasing phosphate; and a closed state (called T state in the allosteric model), in which myosin with bound ADP-phosphate can bind to actin but no phosphate release step can follow. Thus, the transition to the strong binding of myosin with bound ADP or nucleotide-free myosin does not occur in a closed state. These three states are illustrated in Figure 3. Moreover, the closed state is favored against the open state, with an allosteric constant L (the ratio of T state to R state, or closed/open) of ~10. The open state is achieved fully only when the concentration of the myosin head is high enough to shift the allosteric equilibrium from a closed state (T state) toward an open state (R state). When the positions of tropomyosin are determined by electron microscopy in the absence of the myosin head (S1), the position at low $Ca^{2+}$ and high $Ca^{2+}$ concentrations should represent the position at a blocked and a closed state, respectively. The biochemical studies showed that the thin filament is only ~67% blocked even in the absence of $Ca^{2+}$.

## Molecular Organization of Troponin and its $Ca^{2+}$-Induced Changes

Troponin is a target protein of $Ca^{2+}$ signaling and its $Ca^{2+}$-induced changes are important to understanding

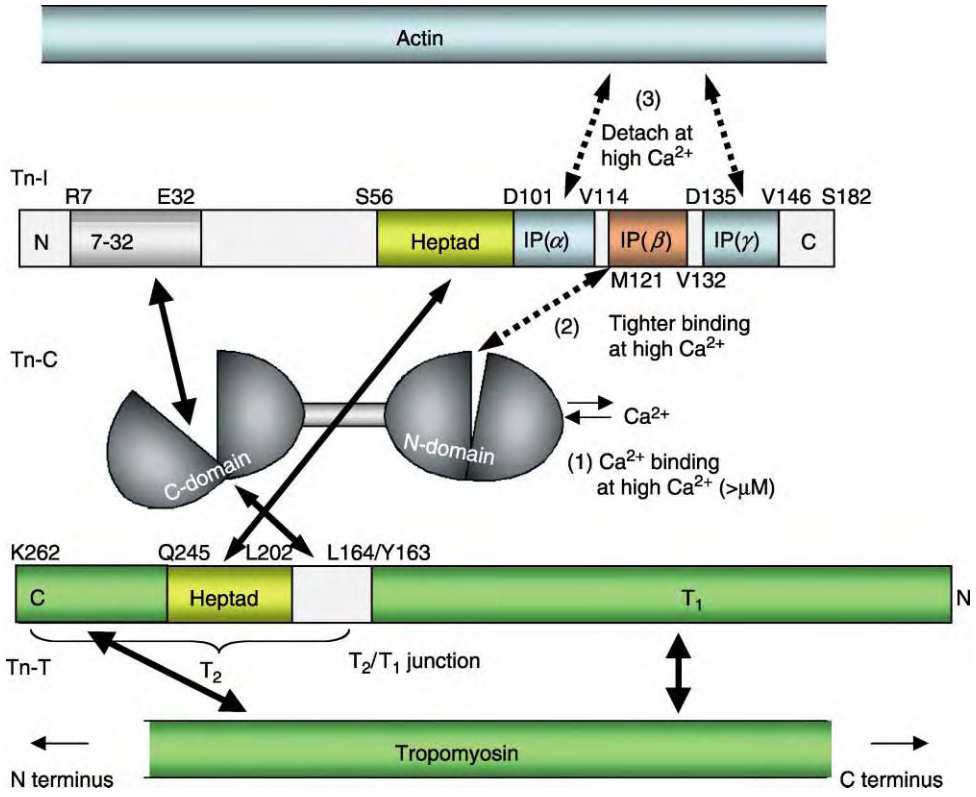



**FIGURE 2** The three steps of $Ca^{2+}$-induced changes in interactions among actin, troponin subunits, and tropomyosin. The interactions indicated by dashed arrows are $Ca^{2+}$-sensitive, whereas those indicated by solid arrows are $Ca^{2+}$-insensitive and constitutive. The numbering shown is for chicken skeletal troponin; skeletal TnI differs from cardiac TnI by ~30 due to the insertion in the N-terminal region of cardiac TnI.

how the muscle contraction is triggered. Such changes have been revealed by ultracentrifugation, X-ray scattering, neutron scattering, circular dichroism, infrared and ultraviolet spectroscopy, fluorescence resonance energy transfer, and chemical cross-linking.

TnC is dumbbell-shaped with two globular domains connected by a long helix. Each domain possesses two EF-hand helix–loop–helix $Ca^{2+}$-binding motifs. The N-terminal domain (N domain in Figure 2) of TnC from skeletal muscle binds two $Ca^{2+}$ specifically but with lower affinity (with a dissociation constant, $K_d$, of a few micromolars), whereas the C-terminal domain (C domain in Figure 2) binds either $Ca^{2+}$ or $Mg^{2+}$ (two $Ca^{2+}/Mg^{2+}$ sites) with higher affinity ($K_d \sim 0.01$ μM). Only one of the EF-hand motifs in the N domain of cardiac TnC binds $Ca^{2+}$. The metal-binding sites of the C domain are occupied always by either $Ca^{2+}$ or $Mg^{2+}$, and the hydrophobic pocket of the C domain is always exposed and binds the N-terminal region (the residues 7–32) of TnI. The corresponding hydrophobic pocket of the N domain becomes open only at high $Ca^{2+}$ (step 1 in the Figure 2).

TnT binds to tropomyosin. Many isoforms have been isolated for TnT. TnT can be divided into $TnT_1$ and $TnT_2$ by chymotryptic cleavage at the carboxyl side of Tyr158 (chicken skeletal TnT), which follows two seryl residues (S156 and S157). $TnT_1$ and the C-terminal region of $TnT_2$ bind to tropomyosin. $TnT_2$ also binds to the C domain of TnC. The primary structures of TnI and TnT show the heptad-repeat pattern – in a heptad the first and the fourth residues are almost always hydrophobic. Two such regions (the residues 56–101 of TnI and 202–245 of TnT) associate and form a coiled coil. Thus, $TnT_2$ binds to tropomyosin, TnI, and TnC.

TnI binds to actin and inhibits the actin-activated ATPase. Its inhibitory activity is neutralized by $Ca^{2+}$-loaded TnC. The residues 97–117 and the peptide corresponding to the residues 96–116 were shown to be responsible for the inhibitory activity and are called the inhibitory region, which approximately corresponds to IP(α) in Figure 2, and the inhibitory peptide (IP), respectively. The numbering shown in Figure 2 is for skeletal troponin, which differs from cardiac TnI by ~30 due to the insertion at the N-terminal region of cardiac TnI. The minimum sequence necessary for inhibition (but not sufficient for full inhibition) is residues 104–115 (GKFKRPPLRRVR), which approximately corresponds to IP(α). There are two other regions with a homologous

sequence, called IP($\beta$) and IP($\gamma$) (Figure 2). The region near residues 117–127, which forms a helix according to the prediction of the secondary structure and the preliminary report of the crystal structure of the cardiac $TnT_2$–TnI–TnC complex at high $Ca^{2+}$ concentrations, follows the inhibitory region and is identified as the target of the hydrophobic pocket in the N domain of TnC; it is called the second TnC-binding site and corresponds approximately to region IP($\beta$).

When $Ca^{2+}$ binds to the N domain of TnC and the hydrophobic pocket becomes open (step 1 in Figure 2), the association between the second TnC binding site of TnI and the N domain of TnC becomes tighter (step 2 in Figure 2). Then, the inhibitory regions IP($\alpha$) and IP($\gamma$) detach from actin (step 3 in Figure 3). This detachment step is key for triggering the activation of muscle contraction. Thus, the N domain of TnC, the C-terminal region of TnI, and actin make up the regulatory machinery. Actin is therefore not just a *target* of $Ca^{2+}$ regulation but is an important *component* of the $Ca^{2+}$ switch.

## $Ca^{2+}$-Induced Troponin Shift on the Thin Filaments

The first event of muscle contraction is the binding of $Ca^{2+}$ to TnC and the detachment of TnI from actin, which is followed by the changes in the location of troponin on the thin filaments. Several parts of TnI have been known to change their positions on the thin filaments. Recent electron cryomicroscopic work shows that troponin changes its shape and shifts toward the inner domain of actin during activation (Figure 3A–B).

The region near Cys117 between the IP($\alpha$) and IP($\beta$) of TnI and the region near Cys133 between the IP($\beta$) and IP($\gamma$) are nearer to the C terminus of actin at low $Ca^{2+}$ concentrations. The details of the troponin shift, however, have been difficult to visualize because troponin does not follow the helical symmetry of actin filament. In 2001, the location of troponin was visualized by the three-dimensional reconstruction from electron cryomicrographs of thin filaments using single-particle analysis. The troponin head (Figure 1B) is gourd-shaped and at high $Ca^{2+}$ concentrations it is located over the inner domain of actin (Figure 3B), whereas at low $Ca^{2+}$ concentrations it is shifted by ~30Å toward the outer domain and bifurcated, with a troponin arm (Figure 3B) covering the N- and C-terminal regions of actin in subdomain 1 (Figure 1B). The troponin arm disappears at high $Ca^{2+}$ concentrations. This might be the consequence of the detachment of the IP($\alpha$) and IP($\gamma$) of TnI from actin. According to the analysis of the data from fluorescence resonance energy transfer experiments, the position of

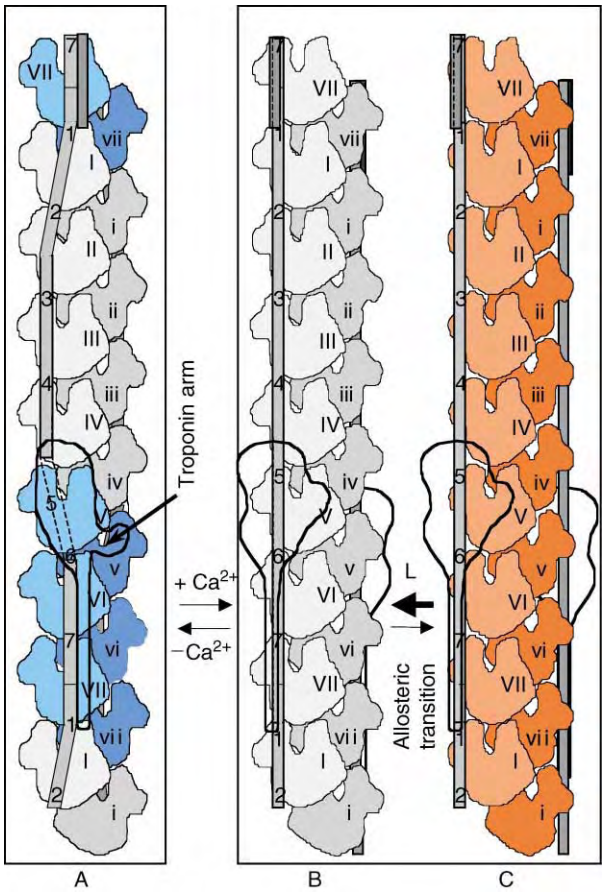



**FIGURE 3** A model for $Ca^{2+}$ regulation. (A) blocked state (T * state); (B) closed state (T state); (C) open state (R state). For simplicity, the two strands of actin are shown untwisted, so that they are parallel, and in different colors. The uppercase roman numerals label the outer domain of actin, and the lowercase ones label the backside of the inner domain. The arabic numerals label the seven segments of tropomyosin, with the segment 5 being the binding site of a troponin head. At high $Ca^{2+}$ concentrations, there are two states, with the T or closed state (B) ~10 times higher in population than the R or open state (C) (L =~10). Myosin favors the transition from the T state to the R state (actin colored orange; myosin can bind and release phosphate to fully support contraction). At low $Ca^{2+}$ concentrations (A), a troponin arm emerges and tropomyosin shifts differentially. Only the actin colored blue is blocked (myosin cannot bind); the actin colored gray is in a closed state (myosin can bind weakly and cannot release bound phosphate). Reprinted from Narita, A., Yasunaga, T., Ishikawa, T., Mayanagi, K., and Wakabayashi, T. (2001). $Ca^{2+}$-induced switching of troponin and tropomyosin on actin filaments as revealed by electron cryomicroscopy. *J. Mol. Biol.*, **308**, 241–261, copyright 2001, with permission from Elsevier.

the C domain of TnC seems to change more than that of the N domain. Presumably, the association between the troponin arm and subdomain 1 of actin pulls the C domain of TnC toward ubdomain 1 at low $Ca^{2+}$ concentrations. At high $Ca^{2+}$ concentrations, however, such interactions disappear and the C domain of TnC returns to its original position over the inner domain of actin.

## $Ca^{2+}$-Induced Tropomyosin Shift

Since the $Ca^{2+}$-induced tropomyosin shift was first proposed, there has been controversy over the locations of tropomyosin, which were determined by assuming helical symmetry. It was found that the thin filaments deviate from helical symmetry; the extent is greater at low $Ca^{2+}$ concentrations than at high $Ca^{2+}$ concentrations. Thus, the locations of tropomyosin were determined by three-dimensional reconstruction without assuming such symmetry.

Tropomyosin follows a smoothly curved path in crystals and in thin filament at high $Ca^{2+}$ concentrations (Figure 1B). At low $Ca^{2+}$ concentrations, however, tropomyosin does not seem to follow a smooth path; it shifts differentially (Figure 3A) at the N-terminal half and the C-terminal third. There is evidence suggesting that the C-terminal third of tropomyosin may not always be in a rigid coiled-coil conformation and is sensitive to the changes in environment (e.g., the changes in troponin). The X-ray crystallographic studies showed that residues 160–220 of tropomyosin are mobile and that its mobility increases at higher temperatures, with the N-terminal half being immobile and almost insensitive to temperature. The region around residue 220 of tropomyosin shows low scores for coiled-coil structure, and the secondary structure prediction of sequence 214–218 of tropomyosin is ambivalent.

The myosin-binding site is located mainly in subdomain 1 in the outer domain of actin. At low $Ca^{2+}$ concentrations, the myosin-binding sites of three actin monomers (colored blue in Figure 3A) out of seven are blocked by the C-terminal third of tropomyosin or troponin. The myosin-binding sites of the remaining four actin monomers (colored gray in Figure 3A), however, are not blocked because the N-terminal half of tropomyosin does not shift much. This is consistent with the biochemical study that found that the thin filament is only 67% blocked even at low $Ca^{2+}$ concentrations. Thus, the simple steric blocking hypothesis is only partially correct.

## Molecular Mechanism of $Ca^{2+}$ Regulation

The relationship between the three states of actin and the location of tropomyosin is presented in Figure 3. When tropomyosin is located on the outer domain, actin monomers are in a blocked state. When tropomyosin is located on the inner domain, there are two states of actin monomers.

Figure 3A–B illustrates the three-dimensional structure of the thin filaments reconstructed from electron cryomicrographs. For clarity, the double helix of thin filament has been untwisted so that two strands are parallel and the strands are colored. The uppercase roman numerals label the outer domains of actin, and lowercase ones label the back side of the inner domains. Arabic numerals label the seven segments of tropomyosin, with segment 5 as the binding site of a troponin head.

At high $Ca^{2+}$ concentrations (Figure 3B), the troponin head looks gourd-shaped and is located over the inner domain of actin (subdomains 3 and 4). Tropomyosin is also located entirely over the inner domain of actin, allowing greater access of myosin to the outer domain, where the myosin-binding site of actin is located, for the generation of force. Thus, all actin monomers are in a closed state (colored gray in Figure 3B).

At low $Ca^{2+}$ concentrations, however, the troponin head shifts toward the outer domain and bifurcates, with a troponin arm covering the N- and C-terminal regions in subdomain 1 of actin. The C-terminal third of tropomyosin also shifts, together with a troponin tail, toward the outer domain of actin and the myosin-binding sites are blocked (colored blue in Figure 3A), but the N-terminal half of tropomyosin shifts only small amount and these myosin-binding sites are still accessible in a closed state (colored gray in Figure 3A). Thus, the myosin-binding sites of only three actin monomers are blocked by tropomyosin and troponin at low $Ca^{2+}$ concentrations.

According to the three-state model, tropomyosin should shift further toward the inner domain of actin to achieve full activation of actin-activated myosin ATPase (Figure 3C). The mutagenesis introduced in residue 230 in subdomain 4 of actin facilitates the actin activation of myosin ATPase in the presence of tropomyosin, with the activation by pure actin being unchanged. Such mutagenesis increases the accessibility of the hydrophobic pocket in subdomain 4 of actin. It is conceivable that tropomyosin shifts further to bind to the exposed hydrophobic pocket to achieve an open state (colored orange).

This model predicts that the full activation of contraction is a two-step process: (1) a $Ca^{2+}$-dependent transition from a blocked state ($T^*$ state) to a closed state (T state) and (2) a $Ca^{2+}$-independent allosteric transition from a closed state (T state) to an open state (R state), facilitated by the binding of myosin to actin. The model also explains why complete relaxation is difficult to achieve in skeletal and cardiac muscles – not all the myosin-binding sites of actin monomers are blocked at low $Ca^{2+}$ concentrations. This may be on purpose; unlike smooth muscles, striated muscles must be always on standby. $Ca^{2+}$ regulation appears to be the result of the delicate balance of interactions among calcium ions, troponin, actin, tropomyosin, myosin, and nucleotides. Such delicateness is consistent with the large number of

reports of mutations that cause familial hypertrophic myocardiopathy.

## See Also the Following Articles

Actin Assembly/Disassembly • Meiosis • Rho GTPases and Actin Cytoskeleton Dynamics

## Glossary

**actin** A globular protein (G-actin, $M_r \sim 42,000$) that forms filaments (F-actin) that bind tropomyosin and troponin. Actin filaments activate myosin ATPase, which supplies the free energy for contraction. The folding pattern of actin is homologous to RNase H.

**myosin (Myosin II)** A motor protein, for example, ATPase, which is activated by an actin filament by approximately 100 times. Myosin consists of two head domains and a tail domain. Tail domains assemble to form thick filaments. A head domain (subfragment 1) can bind to actin and hydrolyze ATP. The myosin head, kinesin, and ras protein share a common folding pattern.

**thin filament** A structure formed by F-actin, tropomyosin, and troponin; native thin filaments also contain nebulin. At high $Ca^{2+}$ concentrations, thin filaments interact with myosin-containing thick filaments to generate force.

**tropomyosin** A fibrous protein ($\sim$400Å, $M_r \sim 35,000 \times 2$) that polymerizes in a head-to-tail manner. When the myosin concentration is low, tropomyosin inhibits actin-activated myosin ATPase. Tropomyosin and troponin are essential for $Ca^{2+}$ regulation.

**troponin** A protein complex formed from troponin-T (TnT, $M_r \sim 31,000$, tropomyosin-binding), troponin-I (TnI, $M_r \sim 21,000$, inhibitory), and troponin-C (TnC, $M_r \sim 18,000$, $Ca^{2+}$-binding). TnT can be divided into $TnT_1$ and $TnT_2$. $TnT_2$, TnI, and TnC form a troponin head, from which a troponin arm emerges at low $Ca^{2+}$ concentrations. $TnT_1$ forms a tail domain.

## Further Reading

Ebashi, S., and Endo, M. (1968). Ca ion and muscle contraction. *Progr. Biophys. Mol. Biol.* **18**, 123–183.

Farah, C. S., and Reinach, F. C. (1995). The troponin complex and regulation of muscle contraction. *FASEB J.* **9**, 755–767.

Huxley, H. E. (1972). Structural changes in the actin- and myosin-containing filaments during contraction. *Cold Spring Harbor Symp. Quant. Biol.* **37**, 361–376.

Maytum, R., Lehrer, S. S., and Geeves, M. A. (1999). Cooperativity and switching within the three-state model of muscle regulation. *Biochemistry* **38**, 1102–1110.

Narita, A., Yasunaga, T., Ishikawa, T., Mayanagi, K., and Wakabayashi, T. (2001). $Ca^{2+}$-induced switching of troponin and tropomyosin on actin filaments as revealed by electron cryo-microscopy. *J. Mol. Biol.* **308**, 241–261.

Squire, J. M., and Morris, E. P. (1998). A new look at thin filament regulation in vertebrate skeletal muscle. *FASEB J.* **12**, 761–771.

## Biography

Dr. Setsuro Ebashi is a Professor Emeritus of the University of Tokyo and a former director of the National Institute of Physiological Sciences. His research interest is the regulatory mechanism of muscle contraction. He holds an M.D and a Ph.D. from the University of Tokyo. He discovered the relaxation induced by $Ca^{2+}$-uptake by sarcoplasmic microsomes, discovered $Ca^{2+}$ regulation of muscle contraction, and isolated troponin.

Dr. Takeyuki Wakabayashi is a Professor of Teikyo University and a Professor Emeritus of the University of Tokyo. His interest is the structural basis of $Ca^{2+}$ regulation of muscle contraction. He holds an M.D. and a Ph.D. from the University of Tokyo. He determined the three-dimensional location of troponin on thin filaments, the myosin-binding site of actin, and the ATP-binding site of myosin; and he showed the differential tropomyosin shift by $Ca^{2+}$.

# Calcium Signaling: NO Synthase

**Zhi-Qiang Wang and Dennis J. Stuehr**
*The Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA*

**Nitric oxide (NO) is an important signal and effector molecule in animal physiology. NO is generated from L-arginine by the NO synthases (NOSs). Three NOSs have been characterized in animals: neuronal NOS (nNOS, type I), cytokine-inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III). Importantly, the activities of all three depend on their binding calmodulin (CaM). CaM binds reversibly to eNOS and nNOS in response to elevated $Ca^{2+}$ concentrations and so their activities are regulated by intracellular $Ca^{2+}$. In contrast, iNOS binds CaM independent of $Ca^{2+}$ concentrations and is therefore continuously active after it is expressed. Because it is unusual for a redox enzyme like NOS to be controlled by a $Ca^{2+}$ binding protein, the NOS–CaM interaction has provided new insight into some aspects of protein structure and function.**

## NO Synthases

To become active each NOS enzyme must assemble into a dimer of two subunits. Each subunit in the NOS dimer contains a N-terminal "oxygenase domain" that binds iron protoporphyrin IX (heme), the cofactor 6R-tetrahydrobiopterin ($H_4B$), and the substrate Arg, and a C-terminal "reductase domain" that binds FMN, FAD, and NADPH. Between oxygenase and reductase domains there is a 20–25 amino acid CaM binding motif (Figure 1).

NO synthesis depends on electron transfer to the heme, and this process only occurs when CaM is bound. In a CaM-bound NOS dimer, the electrons from NADPH load into the FAD and FMN groups in one subunit, and then transfer from the FMN to the heme that is located in the partner subunit (Figure 1). In general, CaM activates electron transfer in NOS at two points: electron transfer into the flavins (intradomain) and between the FMN and heme (interdomain). Intradomain transfer is associated with enhanced catalysis by the NOS reductase domain, as measured by rates of NADPH-dependent ferricyanide or cytochrome c (Cyt c) reduction. CaM activation of the interdomain electron transfer in NOS results in reduction of the ferric heme iron, which is a prerequisite for oxygen binding to the heme and for NO synthesis.

## Calmodulin Interactions with $Ca^{2+}$ and with NOS

CaM is a small $Ca^{2+}$ binding protein (molecular weight 17 kDa) consisting of two similar globular domains (called lobes) that each contain two E–F hands. An E–F hand includes a N-terminal helix (E helix), a centrally located $Ca^{2+}$ coordinating loop, and a C-terminal helix (F helix). Upon $Ca^{2+}$ binding, CaM is able to bind to and activate more than 30 target enzymes, enabling it to regulate numerous second messengers and cell functions. Binding and full activation of target proteins by CaM typically requires occupancy of all four $Ca^{2+}$ binding sites in CaM. The carboxy-terminal lobe contains two high affinity $Ca^{2+}$ binding sites, while the amino-terminal lobe contains two sites with lower $Ca^{2+}$ affinity. Conformational changes that occur in CaM after it binds $Ca^{2+}$ expose its hydrophobic surface residues in order to form critical van der Waals interactions with the hydrophobic face of the target peptide recognition site.

CaM binds to each NOS subunit in a 1:1 stoichiometry with reasonably high affinity. Binding affinity studies with peptides that correspond to NOS CaM recognition sequences show that the three NOSs display different affinities toward CaM with the general order being iNOS $\gg$ nNOS $\approx$ eNOS. The iNOS peptide binds well to CaM both in the presence and absence of $Ca^{2+}$, but eNOS and nNOS peptides bind to the CaM only in the presence of $Ca^{2+}$.

Each lobe of CaM binds to NOS independently and displays different affinities toward NOS. The CaM–nNOS interaction has been investigated using CaM mutants and plant CaM proteins. nNOS displays a high degree of structural specificity toward CaM domains 1, 3, and possibly 4 regarding activation of heme reduction and NO synthesis. The CaM–nNOS interactions are particularly important for electron transfer and involve the latch region of CaM (formed by CaM domains 1 and 3) and a methionine residue in domain 4. These regions of CaM appear to interact with as yet unknown regions on nNOS that are distinct from its CaM binding sequence. $Ca^{2+}$ dissociation from

 © 2004, Elsevier Inc. All Rights Reserved. 

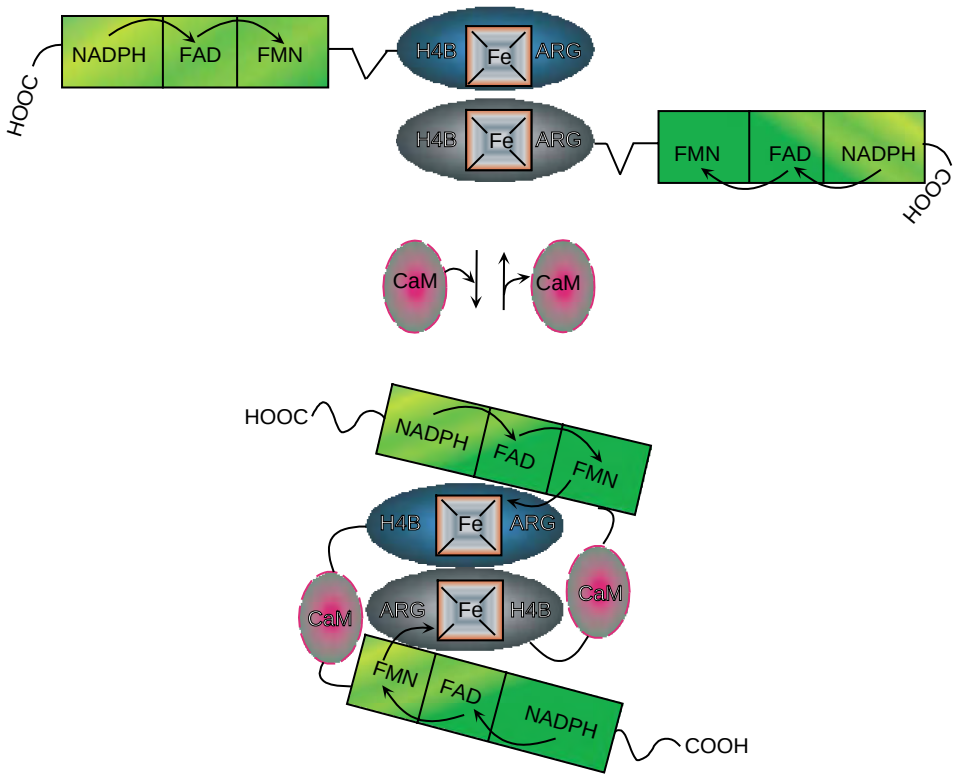



**FIGURE 1** CaM-induced electron transfer in nNOS. CaM binding in response to elevated $Ca^{2+}$ concentrations enables electron transfer between FMN and the heme located in adjacent subunits of the NOS dimer.

NOS-bound CaM has also been investigated. It occurs in two sequential steps: rapid $Ca^{2+}$ dissociation from the N-terminal lobe occurs first and corresponds with inactivation of NO synthesis, followed by a slower $Ca^{2+}$ dissociation from the C-terminal lobe, which leads to the dissociation of CaM from NOS.

Conformational changes occur in the NOS CaM binding peptides when they bind CaM. Typically, the peptides acquire an $\alpha$-helical conformation upon interaction with $Ca^{2+}$ bound CaM. A CaM-induced conformational change in the NOS reductase domain also occurs as indicated by changes in protein and flavin fluorescence and by a change in the trypsin proteolysis pattern.

A crystallographic structure of $Ca^{2+}$-loaded CaM bound to a 20-residue peptide corresponding to the eNOS CaM recognition sequence is available. The structure revealed that the $\alpha$-helical eNOS peptide binds to CaM in an antiparallel orientation through extensive hydrophobic interactions: the N-terminal and C-terminal lobes of CaM wrap around the bound peptide, interacting with the C- and N-terminal halves of the peptide, respectively. Specific CaM residues in the latch region and domain 4 were positioned in a manner consistent with their importance as determined by mutagenesis studies. The crystal structure also suggested a basis for tighter CaM binding in iNOS: because iNOS contains a greater number of hydrophobic residues within its corresponding CaM recognition sequence, it was argued that these would support more extensive van der Waals contacts with CaM and minimize unfavorable solvent exposure of hydrophobic residues in order to favor tighter association between CaM and the iNOS CaM recognition sequence.

## $Ca^{2+}$/CaM Regulation of NOS

How $Ca^{2+}$/CaM regulates NO synthesis and heme reduction in NOS is not entirely clear. CaM binding does not appear to change the thermodynamic driving force for electron transfer in NOS and instead may control the electron transfer primarily via structural rearrangement. CaM binding likely induces a relatively large conformational rearrangement which then enables the FMN subdomain to get close enough to the NOS oxygenase domain heme to make electron transfer between them more efficient.

Some cellular proteins that bind to NOS can regulate its response to $Ca^{2+}$/CaM. These include caveolins, dynamin, the bradykinin $\beta$-2 receptor, and heat shock protein-90. In addition, several different structural elements that are present in NOS regulate its interaction and response to $Ca^{2+}$/CaM (Table I, Figure 2). These

**TABLE I**

**NOS Structural Elements Involved in Its $Ca^{2+}$/CaM Response**

| NOS structural element | Role |
|---|---|
| CaM binding site | Binds CaM to NOS; is inhibitory in the absence of CaM |
| Autoinhibitory loop | Impacts $Ca^{2+}$ concentration response, represses NOS activity in absence of CaM, also required for full CaM response |
| C terminal extension | Impacts $Ca^{2+}$ concentration response, represses NOS activities in absence of CaM, also required for full CaM response |
| Amino acid 484–726 in the FMN subdomain of iNOS | Required for $Ca^{2+}$-independent CaM-binding to iNOS |
| CD2 loop in reductase domain of eNOS | Impacts $Ca^{2+}$ concentration response, represses NOS activities in absence of CaM |
| Phe1395 in FNR subdomain of nNOS | Helps to repress NOS activities in absence of CaM, also required for CaM to fully relieve the repression |
| Phosphorylation sites in eNOS | |
| S1179 | Phosphorylation impacts $Ca^{2+}$ concentration response and increases enzyme activity |
| T497 | Dephosphorylation increases enzyme activity |
| S635 | Phosphorylation may impact enzyme activity |
| S617 | Phosphorylation impacts $Ca^{2+}$ concentration response, no change in enzyme activity |

include the canonical CaM binding site in each NOS and at least four other structural elements within the reductase domain. Initially, it was imagined that the CaM interaction with each NOS isoform might depend solely on its CaM recognition sequence. However, subsequent studies showed that other sequence elements are important. For example, substitution of the eNOS CaM-binding sequence by that of iNOS, and vice versa, yielded proteins whose $Ca^{2+}$ dependence were intermediate between those of iNOS and eNOS. Other studies showed that both eNOS and nNOS, after having their CaM binding sequences replaced with the corresponding sequence from iNOS, still required added $Ca^{2+}$ for full CaM binding and activity. Other more specific deletion studies have identified short regions within the iNOS and eNOS reductase domains that modulate the NOS $Ca^{2+}$ requirement and CaM affinity in a positive or negative manner (Table I). Finally, there are several amino acids that can undergo phosphorylation within the NOS reductase domains and CaM binding sites (Figure 2). Phosphorylation at these individual or combined sites in NOS alters the CaM response with regard to its $Ca^{2+}$ concentration requirement and may also alter the maximal level of enzyme activation that is achieved.

One regulatory element that is unique to NOS is the autoinhibitory loop insert (Figure 2). Constitutive NOSs (for example, eNOS and nNOS) contain a 40–50 amino acid insert in their FMN binding module that is not shared with iNOS or with other related flavoproteins. Similar autoinhibitory loops have been reported in a number of CaM-dependent enzymes, including CaM-dependent protein kinase II, smooth muscle myosin light chain kinase, and calcineurin. Peptides based on the eNOS insert sequence inhibited NOS enzymatic activity by altering their CaM binding. Subsequent studies showed that deleting the insert in eNOS or nNOS decreased their $EC_{50}$ for $Ca^{2+}$, confirming that the insert was inhibitory (i.e., causes the enzyme to require a higher $Ca^{2+}$ concentration to enable its CaM response). Deletion of the autoinhibitory peptide also enables some NO synthesis in the absence of CaM and increases the intrinsic catalytic activity of CaM-bound eNOS. Thus, the insert contributes to the $Ca^{2+}$-dependence of the constitutive NOS, and also regulates the electron transfer between its FMN and heme centers.

How the autoinhibitory insert participates in $Ca^{2+}$/CaM regulation is still unclear. One model has the insert docking on a site in NOSs that impedes both CaM binding and the structural rearrangement that is expected to be required for enzymatic activation. Upon binding, the $Ca^{2+}$/CaM, a conformational change that displaces the autoinhibitory insert from its docking site would then relieve the inhibitory effect and facilitate activation of the enzyme. A two-stage model seems reasonable to explain the $Ca^{2+}$/CaM activation in NOSs: CaM binding is largely determined by the recognition sequence and some other interactions with the enzyme. However, activation of the enzyme is fully dependent on a movement of the autoinhibitory insert, which could only be affected by the $Ca^{2+}$-saturated CaM. The conformational change induced by $Ca^{2+}$/CaM is passed on to the autoinhibitory insert, and this in turn presumably enables the enzyme reductase and oxygenase domains to adopt

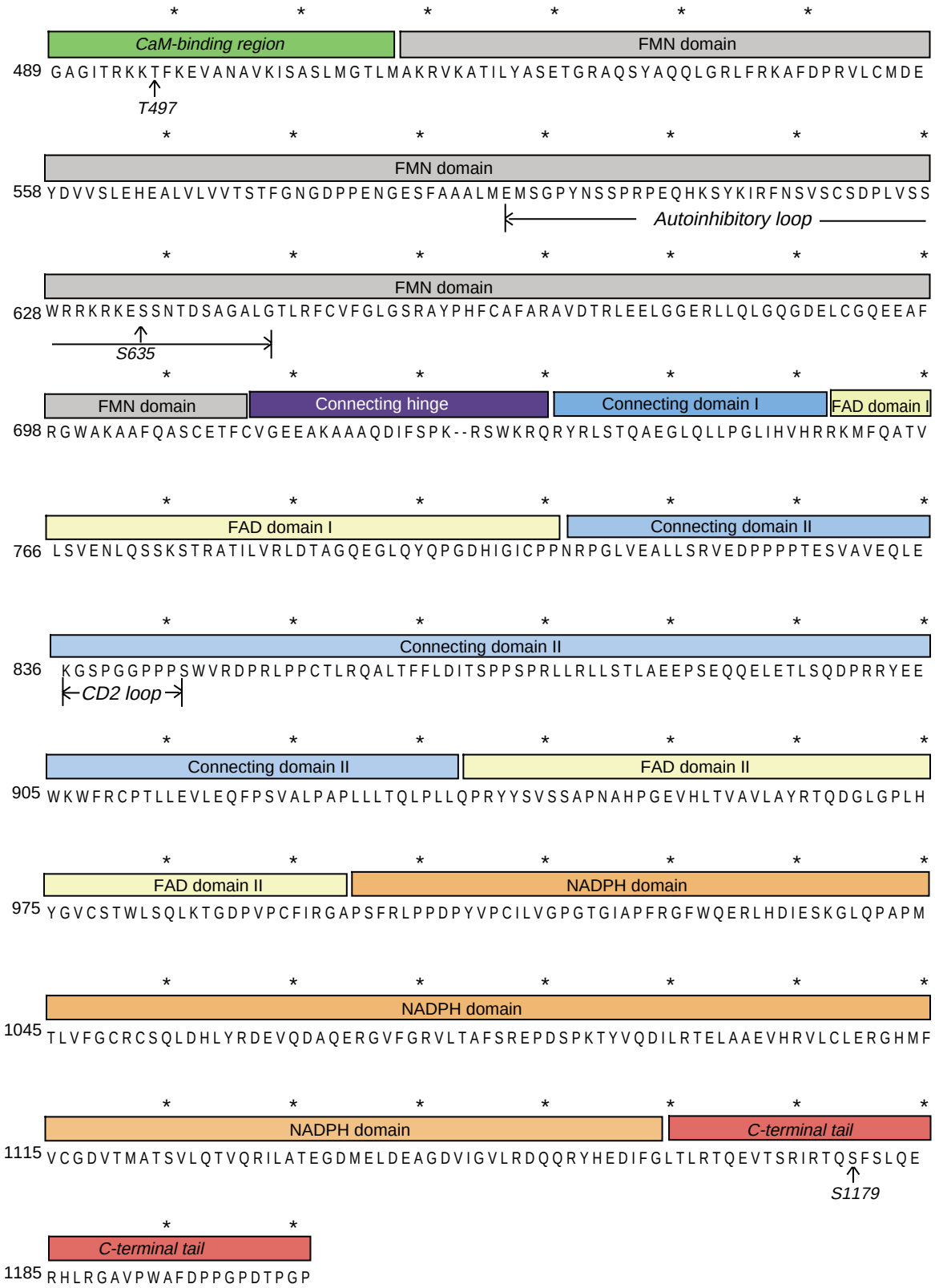



**FIGURE 2** Sequence of eNOS CaM binding site and reductase domain. Colored rectangles indicate subdomains that make up the reductase domain. Structural elements and phosphorylation sites that impact $Ca^{2+}$/CaM regulation of NOS are noted in italics.

conformations that lead to productive electron transfer between the FMN and heme redox centers.

Another regulatory element that is unique to NOS is the C-terminal extension. The protein sequences of NOS reductase domains closely resemble that of NADPH-cytochrome P450 reductase (CPR). However, all NOS isoforms have an additional 20–40 residue extension in their C terminus, forming a "tail" that is absent in CPR. Deletion of 33 or 42 C-terminal residues from nNOS or eNOS speeds electron transfer into the flavins, implying that the C-terminal extension is a negative regulator. Another NOS mutant with a partial deletion of the C-terminal extension (Δ27 eNOS) exhibited a lower $Ca^{2+}$ concentration response and an increase in some of its catalytic activities. Importantly, there is an Akt-dependent phosphorylation site located in the C-terminal tail of the constitutive NOS enzymes that modulates their regulation by $Ca^{2+}$/CaM (Figure 2). Phosphorylation at this site relieves some of the repression attributed to the C-terminal tail in the absence of CaM. It also lowers the $Ca^{2+}$ concentration that is required for CaM binding and increases the NO synthesis activity of eNOS (but not of nNOS). When the autoinhibitory loop is present (as in nNOS and eNOS), the C-terminal tail is also needed to enable full activation in response to $Ca^{2+}$/CaM. This indicates that it functions as a positive regulatory element under this circumstance.

$Ca^{2+}$/CaM regulation of NOS may involve the two regulatory elements in the following manner. In the absence of CaM, the C-terminal extension helps to cover the NADPH-FAD and FMN subdomain interfaces, and this inhibits (but does not completely prevent) the FMN subdomain from transferring electrons to redox partners like cytochrome c or the NOS heme. This repression by the C-terminal tail requires that the enzyme NADPH binding site be occupied. Under this condition, the autoinhibitory insert is positioned in a way that antagonizes CaM binding and prevents the FMN subdomain from interacting productively with the NOS oxygenase domain. Upon CaM binding, the autoinhibitory loop swings away and this conformational change enables interactions between the FMN subdomain and the oxygenase domain that are productive for electron transfer to the heme. Specific interactions between the C-terminal extension and the autoinhibitory insert are likely to occur within the CaM-bound NOS. Such interactions between the two regulatory elements are likely to enable full activation of electron transfer by $Ca^{2+}$/CaM. Thus, the autoinhibitory loop and C-terminal tail repress enzyme function in the absence of CaM, and help to positively modulate function when $Ca^{2+}$/CaM is bound. Other structural elements that are present in the NOS reductase domain (Table I) along with protein phosphorylation events also help to regulate $Ca^{2+}$/CaM binding and to tune its activation of enzyme catalysis.

## See Also the Following Articles

Calcium/Calmodulin-Dependent Protein Kinases • Calcium/Calmodulin-Dependent Protein Kinase II • Cytochrome *c* • Flavins • Heme Proteins • Heme Synthesis • Ligand-Operated Membrane Channels: Calcium (Glutamate) • Nitric Oxide Signaling • Pteridines

## Glossary

**calmodulin** Calcium binding protein that binds to target enzymes in a $Ca^{2+}$-dependent manner and activates their function.

**nitric oxide** Diatomic free radical that is generated from L-arginine by the NO synthases and is widely active in numerous processes in biology.

**reductase domain** Flavoprotein domain of NO synthases that contains bound FAD and FMN and is responsible for transferring electrons from NADPH to the heme group in NO synthase.

## Further Reading

Abu-Soud, H. M., and Stuehr, D. J. (1993). Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10769–10772.

Aoyagi, M., Arvai, A. S., Tainer, J. A., and Getzoff, E. D. (2003). Structural basis for endothelial nitric oxide synthase binding to calmodulin. *EMBO J.* **22**, 766–775.

Hemmens, B., and Mayer, B. (1998). Enzymology of nitric oxide synthases. *Methods Mol. Biol.* **100**, 1–32.

James, P., Vorherr, T., and Carafoli, E. (1995). Calmodulin-binding domains: Just two faced or multi-faceted? *Trends Biochem. Sci.* **20**, 38–42.

Nishida, C. R., Knudsen, G., Straub, W., and Ortiz de Montellano, P. R. (2002). Electron supply and catalytic oxidation of nitrogen by cytochrome P450 and nitric oxide synthase. *Drug. Metab. Rev.* **34**, 479–501.

Persechini, A., White, H. D., and Gansz, K. J. (1996). Different mechanisms for $Ca^{2+}$ dissociation from complexes of calmodulin with nitric oxide synthase or myosin light chain kinase. *J. Biol. Chem.* **271**, 62–67.

Roman, L. J., Martasek, P., and Masters, B. S. (2002). Intrinsic and extrinsic modulation of nitric oxide synthase activity. *Chem. Rev.* **102**, 1179–1190.

## Biography

Zhi-Qiang Wang is a Research Associate in the Department of Immunology at the Lerner Research Institute, Cleveland Clinic Foundation, in Ohio. She holds a Ph.D. from Fudan University (People's Republic of China) and served as a postdoctoral fellow at the Cleveland Clinic. Her principal research interests are the structure and function relationships of enzymes.

Dennis Stuehr is a Professor of Molecular Medicine and a member of the Department of Immunology at the Lerner Research Institute, Cleveland Clinic, in Cleveland, Ohio. He holds a Ph.D. from the Massachusetts Institute of Technology and received his postdoctoral training at Cornell University Medical School.

# Calcium Transport in Mitochondria

**Rosario Rizzuto**
*University of Ferrara, Ferrara, Italy*

**Marisa Brini**
*University of Padova and Venetian Institute of Molecular Medicine (VIMM), Padova, Italy*

**Mitochondria have long been known to accumulate $Ca^{2+}$ down the electrical gradient established by the respiratory chain. Recent work has shown that, despite the low affinity of their $Ca^{2+}$ transporters, $Ca^{2+}$ uptake into mitochondria always follows the stimulation of cells with agonists causing an increase of cytoplasmic $Ca^{2+}$ concentration. This process can modulate cellular events as diverse as aerobic metabolism, cytoplasmic diffusion of $Ca^{2+}$ signals, and induction of apoptotic cell death.**

## The Origins and the Fundamental Principles

Studies of $Ca^{2+}$ transport in isolated mitochondria began in the 1960s when evidence was provided that isolated, respiring mitochondria accumulate $Ca^{2+}$ in the presence of inorganic phosphate. $Ca^{2+}$ uptake was inhibited by respiratory chain blockers, but not by the ATP synthesis inhibitor oligomycin, and during $Ca^{2+}$ uptake no ADP phosphorylation took place. In the presence of ATP no respiratory chain activity was necessary. Thus, the process of $Ca^{2+}$ uptake was regarded as an alternative pathway to ADP phosphorylation for the use of the respiratory chain energy. In this scenario, mitochondria were thought as large stores of $Ca^{2+}$, which could be accumulated together with inorganic phosphate and precipitate in the matrix, leaving the matrix $Ca^{2+}$ concentration virtually unchanged.

The general picture became clearer when the chemiosmotic theory solved the mechanism allowing mitochondria to store the energy obtained from metabolite breakdown and couple it to ATP synthesis. Indeed, the concept that the respiratory chain establishes a gradient ($\Delta\mu_H$), given by the Nernst equation $\Delta\mu_H = zF\Delta\psi + RT\ln[H^+]_i/[H^+]_o$, has major implications for $Ca^{2+}$ transport and distribution. The gradient, composed of an electrical potential ($\Delta\psi$) and a concentration ratio ($\Delta$pH), results by pumping protons across the ion-impermeable inner membrane. In actively respiring mitochondria, considering the buffering capacity mostly provided by weak acids, the gradient is supposed to be maintained mostly in the form of electrical gradient across the inner membrane ($\sim$180 mV). This implies a strong thermodynamic force in favor of the accumulation of cations (in the case of a divalent cation, such as $Ca^{2+}$, thermodynamic equilibrium is attained when in the matrix the concentration is $\sim 10^6$ higher than in the inter-membrane space). Based on these simple considerations, much attention was focused in the 1960s and 1970s on the capacity of mitochondria to accumulate $Ca^{2+}$, and on the biochemical and thermodynamic properties of this transport. Through the contribution of numerous laboratories and a large body of experimental work carried out in isolated, respiring mitochondria, these organelles were shown to possess separate accumulation and release pathways for the cation along with defined characteristics. Accumulation was shown to depend on the activity of an electrogenic "uniporter," which transports $Ca^{2+}$ down the electrical gradient established by the respiratory chain. Inhibition of the respiratory chain or collapse of the electrical gradient (e.g., by the use of a protonophore) abolishes the capacity of mitochondria to accumulate $Ca^{2+}$. $Ca^{2+}$ uptake is also directly inhibited by the compound Ruthenium Red (or its recently identified subcomponent Ru360) or lanthanides. Unfortunately, none of the inhibitors is highly specific, thus no selective tool has aided the purification of the transporter. Indeed, through the years this as well as the other mitochondrial $Ca^{2+}$ transporters were not purified, and thus, despite the renewed interest, no molecular information on this process is available. As for the release pathways, two sets of exchangers have been shown to extrude $Ca^{2+}$ from mitochondria: a $Na^+/Ca^{2+}$ exchanger, mostly expressed in excitable cells (muscle and brain) and a $H^+/Ca^{2+}$ exchanger, that represents the prevailing route in most other tissues. Both exchangers (although with different $K_d$) are inhibited by verapamil, diltiazem and other $Ca^{2+}$ channel blockers.

 © 2004, Elsevier Inc. All Rights Reserved. 

More recently, much attention has been drawn to the potential role of a large-conductance channel, commonly referred to as the permeability transition pore (PTP). It is supposed to be a multiprotein complex (the putatively essential components being the voltage dependent anion channel, VDAC, of the outer membrane, the adenine nucleotide transporter, ANT, of the inner membrane and cyclophilin D) activated by various pathophysiological conditions (e.g., $Ca^{2+}$ increases in the mitochondrial matrix and oxidation of critical cysteins). However, its role in mitochondrial $Ca^{2+}$ homeostasis still remains elusive: it appears to play no role in $Ca^{2+}$ efflux (as expected, considering that, unless an almost complete collapse of the electrical gradient occurs, the driving force is for $Ca^{2+}$ accumulation, not release) and there is no clear evidence for its participation in mitochondrial $Ca^{2+}$ uptake.

## The Middle Ages

In the 1980s mitochondrial participation in intracellular $Ca^{2+}$ homeostasis rapidly lost support, as the focus in calcium signaling was largely diverted to the endoplasmic reticulum (ER). In these years, the chain of events that couples the stimulation of plasma membrane receptors to the induction of a rise in cytoplasmic $Ca^{2+}$ concentration was clarified, and it became clear that the ER and not mitochondria, were the main sites of action. Indeed, stimulation of receptors coupled through a $G_q$ protein to the activation of phospholipase C induces the hydrolysis of the plasma membrane phospholipid phosphatidylinositol 4,5bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5trisphosphate (IP3). IP3 diffuses into the cytosol and interacts with $Ca^{2+}$-permeant channels of the ER membrane, causing their opening and the release of $Ca^{2+}$ into the cytoplasm. The ER (and its specialized version of muscle cells, the sarcoplasmic reticulum, SR) was shown to be endowed with a molecular repertoire allowing it to act as a highly reactive intracellular $Ca^{2+}$ store: pumps (that accumulate $Ca^{2+}$ at the expense of ATP hydrolysis, and maintain a standing gradient between the lumen and the cytosol), low-affinity $Ca^{2+}$ binding proteins (that increase the net amount of cation that can be accumulated, but promptly make it available when the concentration in the lumen decreases), and channels (opening, with different mechanisms, after stimulation of plasma membrane receptors).

What was left for mitochondria? In principle, given the large thermodynamic force for $Ca^{2+}$ accumulation, they could take up most of the $Ca^{2+}$ released by the ER. However, in the same years the availability of powerful and versatile $Ca^{2+}$ indicators, the intracellularly trappable fluorescent dyes (e.g., quin2, fura-2, etc.) allowed for the careful estimation of cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) of living cells and further reduced the possible roles of mitochondria. Indeed, these indicators showed that both at rest (0.1 μM) and upon stimulation (1–3 μM) the $[Ca^{2+}]_c$ is well below the affinity of the mitochondrial uniporter, and thus very little $Ca^{2+}$ is supposed to be accumulated in mitochondria during the brief pulse of a physiological stimulation. Thus, the general consensus incorporating this series of information was that mitochondria acted as a low-affinity sink that could accumulate large $Ca^{2+}$ loads, but only if the cell was exposed to challenges (most likely pathological events) that induced sustained, large $[Ca^{2+}]_c$ increases. In other words, mitochondria were mostly intended as salvage mechanisms against deleterious $Ca^{2+}$ overloads.

## The Renaissance

In this situation, the only opportunity for mitochondria to reobtain credit in the calcium field was to directly demonstrate in intact, living cells changes in mitochondrial $[Ca^{2+}]$ occurring in physiological conditions. This became possible in the early 1990s when novel experimental tools allowed to specifically measure the $Ca^{2+}$ concentration in the mitochondrial matrix ($[Ca^{2+}]_m$): positively charged fluorescent indicators, which are largely accumulated in the mitochondria and molecularly engineered chimeras of the $Ca^{2+}$-sensitive photoprotein aequorin that include mitochondrial targeting sequences, and are thus exclusively localized in the mitochondrial matrix. Using these probes, it was possible to demonstrate that, in a variety of cell systems (ranging from epithelial cells to skeletal and cardiac myocytes, from hepatocytes to neurons), the $[Ca^{2+}]_c$ rises evoked by physiological stimulations are always paralleled by rapid $[Ca^{2+}]_m$ increases, which reach values well above those of the bulk cytosol (up to ~500 μM in chromaffin cells). The obvious discrepancy between this prompt response and the low affinity of the calcium uniporter was reconciled by the demonstration that mitochondria are exposed to microdomains of high $[Ca^{2+}]$ that largely exceed the values reported in the bulk cytosol and meet the low affinity of the uniporter. This is achieved through a close interaction between the mitochondria and the ER, the intracellular $Ca^{2+}$ store, which could be directly demonstrated using targeted chimeras of a fluorescent recombinant protein (GFP) and a high-resolution imaging system. A consequence of this morphological arrangement is the capacity of mitochondria to "sense" the microenvironment at the mouth of the IP3-sensitive channel (and/or the plasma membrane $Ca^{2+}$ channels), and thus the high $[Ca^{2+}]_c$ generated by their opening upon cell stimulation (Figure 1).

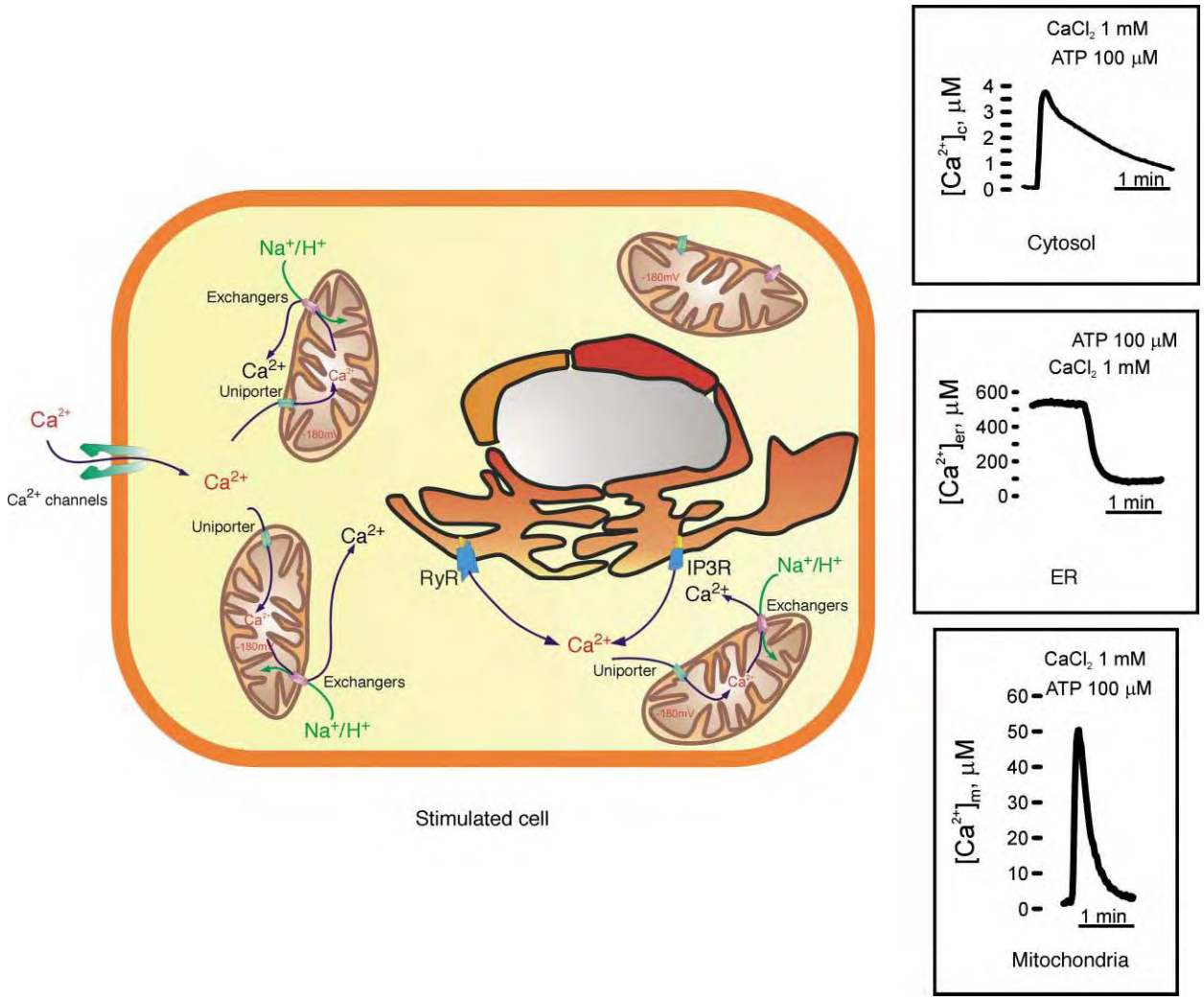



**FIGURE 1** Mitochondrial $Ca^{2+}$ transport. Cell stimulation induces the opening of plasma membrane $Ca^{2+}$ channels and ER $Ca^{2+}$ channels (IP3R and RyR), which, in turn, generates "hot spots" of $Ca^{2+}$ concentration. Mitochondria located in the $Ca^{2+}$ channels proximity could rapidly take up $Ca^{2+}$ since their low-affinity uniporter could experience high local $Ca^{2+}$ concentrations. The three traces represent measurements of $[Ca^{2+}]$ in the three cellular compartments shown, performed with suitably targeted aequorin.

## One (or Many) Roles?

What is the functional significance of the re-evaluated mitochondrial $Ca^{2+}$ transport? An obvious answer again stems from biochemical work by Denton, McCormack and Hansford in the 1960s, who could demonstrate that three key metabolic enzymes (the pyruvate, $\alpha$-ketoglutarate and isocitrate dehydrogenases) are activated by $Ca^{2+}$, by different mechanisms: in the case of pyruvate dehydrogenase through a $Ca^{2+}$-dependent dephosphorylation step, and in the latter two cases through the direct binding of $Ca^{2+}$ to the enzyme complex. Thus, the extension of a $Ca^{2+}$ signal originated in the cytoplasm to the mitochondria would serve the purpose of transmitting an activatory signal to the energy powerhouse of the cell. In conjunction with the triggering of energy-consuming processes in the cytosol (contraction, secretion, etc.), mitochondrial dehydrogenases are stimulated, thus adapting aerobic metabolism to the increased needs of an active cell. Using the luciferase-based probe for ATP, it was possible to demonstrate that a rise in mitochondrial ATP levels parallels the $[Ca^{2+}]_m$ increase evoked by cell stimulation, and strictly depends on the $[Ca^{2+}]_m$ rise; if the latter is prevented by the use of $Ca^{2+}$ chelators, such as 1,2-Bis(2-aminophenoxy) ethane-N, N, N′, N′-tetracetic acid (BAPTA), the [ATP] rise does not occur.

However, recent work by different groups has clarified that the role of mitochondrial $Ca^{2+}$ uptake is not limited to the control of organelle function, but has a direct impact on the $Ca^{2+}$ signals evoked by agonist stimulation in the cytosol. Two different mechanisms concur in this effect. The first occurs in the microdomains where mitochondria and ER get in

close contact. Here, the efficiency of mitochondrial $Ca^{2+}$ accumulation accounts for the rapid clearing of the high $[Ca^{2+}]$ at the mouth of the release channel of the ER, and thus reduces the (positive or negative) feedback effect of the cation on the channel itself. Such a mechanism has been shown in a variety of experimental systems ranging from Xenopus oocytes (in which the diffusion properties of $Ca^{2+}$ waves correlates with the energization state of mitochondria) to mammalian cells such as hepatocytes and glial cells, where it was demonstrated that the kinetics of $Ca^{2+}$ release from the ER, and thus the spatio-temporal properties of the $[Ca^{2+}]_c$ rise are influenced by the process of mitochondrial $Ca^{2+}$ uptake.

The second mechanism by which mitochondrial $Ca^{2+}$ uptake affects cytosolic $Ca^{2+}$ signals has been demonstrated in pancreatic acinar cells, which are endowed with a defined polarized morphology and the occurrence of cellular $Ca^{2+}$ signals with different cellular locations and physiological consequences.

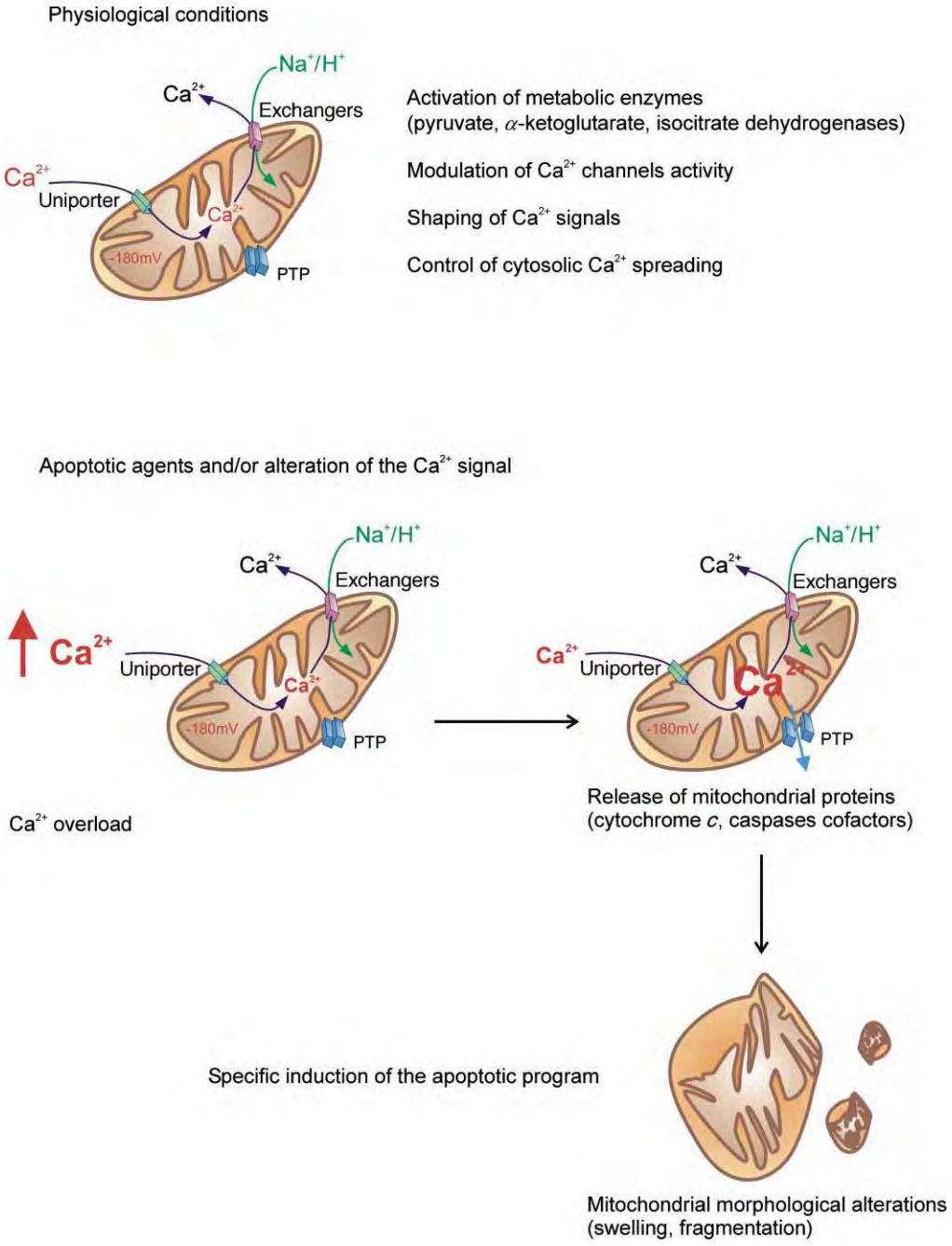



**FIGURE 2** Physiological and pathological role of mitochondrial $Ca^{2+}$ signaling.

At lower doses of agonists (e.g., colecystokinin) the $[Ca^{2+}]_c$ rise is restricted to the apical pole and causes granule secretion, at higher doses the $[Ca^{2+}]_c$ rise extends to the basal pole (where the nucleus is located), thus causing activation of gene expression and long-term alterations of cell function. The restriction mechanism was shown to be dependent on $Ca^{2+}$ uptake by mitochondria clustered below the apical region that act as a "firewall" preventing the spread of the $[Ca^{2+}]_c$ rise. When the mitochondrial "belt" is overwhelmed (e.g., upon intense stimulation, or when the $Ca^{2+}$ uptake capacity of mitochondria is experimentally impaired), then the $Ca^{2+}$ signal can freely diffuse to the rest of the cell.

Finally, in the past years a more "dangerous" role for mitochondrial $Ca^{2+}$ uptake has emerged. Work from various labs has revealed that the alteration of the $Ca^{2+}$ signal reaching the mitochondria and/or the combined action of apoptotic agents or pathological conditions (e.g., oxidative stress) can induce a profound alteration of organelle structure and function. As a consequence, proteins normally retained in the organelle (such as an important component of the respiratory chain, cytochrome *c*, as well as newly discovered proteins, such as AIF and Smac/Diablo) are released into the cytoplasm, where they activate effector caspases and drive cells to apoptotic cell death. Different effects have been described: in hepatocytes, upon treatment with suboptimal doses of the lipid mediator of apoptosis ceramide, the repetitive spiking of cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) rather than triggering the activation of matrix dehydrogenases (and thus the stimulation of aerobic metabolism), causes the opening of the permeability transition pore, the swelling of mitochondria, and the release of cytochrome *c*. Thus, a mechanism of "coincidence detection" of physiological agonists and apoptotic stimuli allows the specific induction of the apoptotic program (the mitochondria acting as the site where this "differential decoding" is operated). In addition, apoptotic stimuli can induce the impairment of the $Ca^{2+}$ clearing mechanism of the cell (the plasma membrane $Ca^{2+}$-ATPase) thus potentiating $Ca^{2+}$ loading. In this scenario, it can be easily understood how the partial depletion of $Ca^{2+}$ from the ER, induced by the oncogene Bcl-2, has a protective effect toward some apoptotic stimuli (those involving mitochondria in the signaling pathway). Both in neurons and in hepatic cells it was observed that apoptotic signals cause mitochondrial $Ca^{2+}$ overload, thus resulting in apoptotic morphological and functional alterations. A general scheme of the physiological role of mitochondrial $Ca^{2+}$ signaling, and the potential alterations occurring in pathological conditions (modifying the final outcome) is summarized in Figure 2.

## Conclusions

Recent work has greatly re-evaluated mitochondrial $Ca^{2+}$ homeostasis as a key event not only for the maintenance of ion balance within the organelle (avoiding deleterious short-circuitry of the chemiosmotic machinery), but also as a control mechanism for physiological and pathological processes occurring in mitochondria, or activated by mitochondrial factors. Thus, both in basic and applied research there is an expanding interest on this topic, that contrasts with the paucity of molecular information. Neither the transporters nor the regulatory elements (and the crosstalk with other signaling routes) have been characterized, leaving a stimulating challenge (and much work to do) for scientists joining the field.

## See Also the Following Articles

ATP Synthesis in Plant Mitochondria: Substrates, Inhibitors, Uncouplers • Calcium Signaling: Cell Cycle • Cell Death by Apoptosis and Necrosis • ER/SR Calcium Pump: Function • ER/SR Calcium Pump: Structure • Membrane Transporters: $Na^+Ca^{2+}$ Exchangers • Phosphatidylinositol Bisphosphate and Trisphosphate • Voltage-Sensitive $Ca^{2+}$ Channels

## Glossary

**apoptosis** A type of cell death, characterized by specific morphological changes (i.e., chromatin condensation, DNA damage, and shrinkage of the cells) and utilized by organisms to regulate the growth and the development of tissues, organs, etc. Also called "programmed cell death."

**ATP** Adenosine trisphosphate, the biochemical molecule utilized by the cells as energy source.

**caspases** Family of proteins responsible for the degradation of specific proteins that contain recognizing sequences. Their activation leads to signals of programmed cell death.

**G protein** Protein associated to specific hormone receptor of the plasma membrane and responsible for the conversion of extracellular signals in intracellular messages.

**respiratory chain** A series of multisubunits proteins located in the inner mitochondrial membrane responsible for the electron and proton transfer necessary to generate the electrochemical gradient used for ATP production.

## Further Reading

Carafoli, E. (2003). Historical review: Mitochondria and calcium: Ups and downs of unusual relationship. *Trends Biochem. Sci.* **28**, 175–181.

Carafoli, E., Santella, L., Branca, D., and Brini, M. (2001). Generation, control, and processing of cellular calcium signals. *Crit. Rev. Biochem. Mol. Biol.* **36**, 107–260.

Duchen, M. R. (1999). Contributions of mitochondria to animal physiology: From homeostatic sensor to calcium signalling and cell death. *J. Physiol.* **16**, 1–17.

Green, D., and Kroemer, G. (1998). The central executioners of apoptosis: Caspases or mitochondria? *Trends Cell. Biol.* **8**, 267–271.

MacCormack, J. G., Halestrap, A. P., and Denton, R. M. (1990). Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol. Rev.* **70**, 391–425.

Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993). Microdomains of high $Ca^{2+}$ close to inositol-triphosphate sensitive channels are sensed by neighbouring mitochondria. *Science* **262**, 744–747.

Rizzuto, R., Bernardi, P., and Pozzan, T. (2000). Mitochondria as all-round players of the calcium game. *J. Physiol.* **529**, 37–47.

## Biography

Rosario Rizzuto is a Professor of General Pathology at the University of Ferrara. He developed a new method for measuring $Ca^{2+}$ concentration in specific cell domains, based on the targeting of the $Ca^{2+}$-sensitive photoprotein aequorin. His research interest is the role of calcium ions as intracellular second messengers with special emphasis on the mechanism and functional role of mitochondrial $Ca^{2+}$ homeostasis.

Marisa Brini is Assistant Professor of Biochemistry at the University of Padova. Her main interest is the study of calcium signaling defects occurring in human genetic diseases and/or transgenic models affecting the homeostasis machinery of muscle. These include malignant hyperthermia, mitochondrial disorders, and cardiopathies due to mutations in the $Ca^{2+}$ ATPase modulator phospholamban.

# Calcium Waves

**Lionel F. Jaffe**
*Marine Biological Laboratory, Woods Hole, Massachusetts, USA*

**Calcicum waves are actively propagated increases in intracellular calcium that carry signals within all organisms above the bacterial level. They occur in four main classes with characteristic speeds that vary over a range of a billionfold. Fast calcium waves move at 10 to 30 micrometers per second along endoplasmic reticula and include fertilization waves and brain injury waves. Slow calcium waves move at 0.1 to 1 micrometer per second and are surface contraction waves such as those that cleave dividing cells. Ultrafast calcium waves move at 10 to 40 centimeters per second along cell membranes and are electrical ones which underlie calcium action potentials in nerves and muscles. Ultraslow calcium waves move at 0.1 to 10 nanometers per second during development and include waves of DNA replication and of floret formation. Their mechanism is unknown.**

## Fast Calcium Waves

### History, Roles, and Visualization

Calcium waves occur in four main classes with characteristic speeds that vary over a range of a billionfold. This speed spectrum is shown in Figure 1.

Calcium waves were discovered in 1978 by Gilkey *et al.* as the giant 10 μ/s fast wave or tsunami that crosses a medaka fish egg as it is fertilized and serves to start its development or activate it. This was done by injecting the eggs with the chemiluminescent calcium reporter, aequorin, and observing the wave of luminescence with an ultralow light imaging system (Figure 2). The American, Ernest Just first grasped the significance of fertilization waves and first measured their speeds accurately (in sea urchin eggs) in 1919. Just believed that such waves were restricted to the cell cortex, but they are now known to traverse the whole cytoplasm including the nucleus. The role and speed of such fertilization waves through medaka eggs was discovered by Tok-io Yamamoto during the Second World War in Japan. Moreover, he correctly predicted that they would prove to be calcium waves. Whereas sea urchin and fish eggs are activated by a single fast calcium wave, many other eggs – including human ones – can only be fully activated by a long series of fast calcium waves that start every few minutes. Aequorins still provide the best way to visualize calcium waves because these reporters are nondisturbing and provide quantitative information over a far larger dynamic range than the more widely used fluorescent indicators. Other roles of fast calcium waves include the control of contraction within muscle cells isolated from whole hearts, the control of contraction within isolated uterine muscle cells, the control of spreading depression or convulsion in the injured brain, the control of neuroglial waves in various vertebrate retinas, and the control of secretion in isolated rat livers.

Among brain injury waves are those that propagate migraine attacks and stroke. As early as 1941, the propagation of fast waves through the visual cortex of the brain during migraine attacks was inferred by Karl Lashley from the wave of visual blurring that sometimes accompanies migraine attacks.

### Mechanisms

The initiation of fast calcium waves is best understood during fertilization. The interaction of eggs and sperm starts when contact of the sperm with the jelly coat around the egg induces a flow of calcium ions into the sperm from the medium. The influx ceases only when the sperm dies of calcium poisoning approximately 1 hour later. When the gametes fuse, this influx is diverted into the egg to help initiate a calcium wave. In most creatures, initiation is also speeded up by small molecules (sometimes called second messengers), such as nicotinic acid adenine dinucleotide phosphate (NAADP) or nitric oxide, that diffuse into the egg along with calcium ions.

Fast calcium waves are propagated within cells by a reaction–diffusion cycle or cycles that travel along the endoplasmic reticulum (ER). In the best understood of these cycles, the only reaction is one that opens calcium channels within the ER membrane. Calcium ions then move through such a channel from the lumen of the ER to the region just outside it. There they diffuse along the outside of the ER to other calcium channels a few microns away so as to induce their own release; a reaction called calcium-induced calcium release (CICR). In this way, a fast calcium wave is relayed from channel to channel so as to traverse a cell. Successful relay also requires a high enough level of channel sensitizers.

 © 2004, Elsevier Inc. All Rights Reserved. 




**FIGURE 1** Main classes of natural calcium waves based on speeds at room temperature. Fast waves through active cells refers to all such waves in this speed class except for the slightly slower ones that activate eggs and are marked f. Fast waves are reaction–diffusion waves propagated by the short-range diffusion of calcium ions between relay points in the endoplasmic reticulum (ER). Slow waves are a second coherent class of calcium waves and seem to be stretch propagated. Ultrafast waves are electrically propagated waves or action potentials that are primarily dependent on an influx of calcium, rather than sodium, ions. Ultraslow waves are developmental. Reproduced with permission from L.F. Jaffe (1999). *BioEssays* **21**, 657–677. Copyright 1999, John Wiley & Sons.

In various cells these include cyclic AMP (cAMP), inositol trisphosphate ($IP_3$), cyclic adenosine 5′-diphosphate-ribose (cADPR), as well as NAADP. One way that fast calcium waves are carried between cells is by the diffusion of calcium ions through minute cytoplasmic bridges called gap junctions. Reaction–diffusion waves can also be seen in dishes containing a complex mixture of nonbiological chemicals such as the brominated ones that support the Bielozov–Zhabotinsky (BZ) reaction.

However, they may also be propagated along the interiors or lumens of ER strands by a reaction–diffusion cycle in which calcium induces its own release from a luminal calcium-binding protein such as a calsequestrin, a calreticulin, or a calcistorin/PDI. The two cycles mutually reinforce one another like the riders of a tandem bicycle; this is called a tandem wave mechanism.

## Slow Calcium Waves

### History, Roles, and Visualization

Slow calcium waves (like fast ones) were discovered in medaka fish eggs with the aid of aequorin. They were seen as two successive waves, both of which move at 0.5 μm/s within the forming second furrow of the cleaving medaka egg. The first of these accompanies elongation of the second furrow, whereas the second accompanies the subsequent, sequential apposition, or zipping together, of the newly formed blastomeres. This discovery was made in 1991 and the results of calcium buffer injections into Xenopus eggs (which likewise exhibit slow calcium waves during cleavage) soon showed that these waves do more than accompany furrowing. They are needed for it to start, to continue, and to remain. Moreover, by 1998, 30 apparently similar slow surface contraction waves had been recognized. An especially vivid one is the repeated gross contractile waves that traverse the barnacle egg from pole to pole before its first mitotic division. Its discoverers called these barnacle egg waves peristaltic in the plausible belief that they serve to drive morphogens to the vegetal pole via intracellular peristalsis. Another remarkable one is the surface contraction wave that accompanies primary neural induction in the axolotl egg.

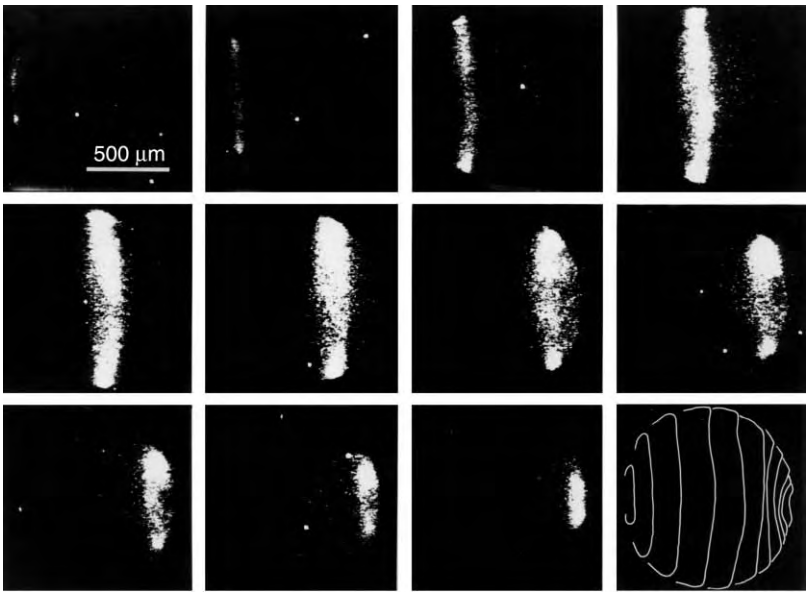



**FIGURE 2** A free calcium wave propagating across a sperm-activated medaka egg. Successive photographs are 10 seconds apart. The last frame is a tracing showing the leading edges of the 11 illustrated wave fronts. Reproduced from Gilkey, J. C., Jaffe, L. F., Ridgway, E. B., and Reynolds, G. T. (1978). A free calcium wave traverses the activating egg of the medaka, *Oryzias latipes*. *J. Cell Biol.* **76**, 448–466, by copyright permission of the Rockefeller University Press.

### Mechanisms

Slow calcium waves are thought to be propagated mechanically. Local surface contraction opens stretch-activated calcium channels in the subsurface ER of nearby regions. This releases calcium ions, which induces contraction in these nearby regions and thereby relays the wave. If the initial contraction is along a line, then the wave spreads along a line as in a cleavage furrow; however, if the initial contraction acts to shrink a small disk, then the wave spreads over the entire surface, as in the precleavage surface contraction waves of axolotl eggs.

## Ultrafast Calcium Waves

Action potentials are electrical waves that are propagated by voltage-gated ion channels in the plasma membranes of nerve, muscle, and other cells. In the best-understood action potentials, sodium ions flow in through these ion channels to extend the electrical field along a cell; however, in another important class of action potentials, calcium ions flow in; these are called calcium action potentials or ultrafast calcium waves. The speeds of calcium action potentials have been measured along neurons within systems that range from jellyfish up to guinea pig brains, along muscles that range from moth hearts up to guinea pig hearts and even along an insectivorous plant. Unlike sodium action potentials, whose speeds vary over a thousandfold range, calcium action potential speeds vary over a range of approximately 10–40 cm/s (at 20°C) and thus over only a fourfold range. Moreover, unlike the speeds of sodium action potentials, the speeds of calcium ones are unrelated to cell diameter.

Why do calcium action potentials, or ultrafast calcium waves, have such a limited range of speeds? Perhaps evolution has driven them to be the fastest waves of calcium influx that avoid subsurface poisoning of the cell.

## Ultraslow Calcium Waves

Ultraslow calcium waves are the least understood class of calcium waves. They include waves of formation of the morphogenetic furrow in developing Drosophila eyes, of floret formation in sunflowers, of DNA replication in certain protozoa, and of growth-cone-like bulges along certain growing mammalian brain neurons, as well as, perhaps, waves of cell division in rat lenses as they heal after a wound. The propagation mechanism of ultraslow calcium waves is completely unknown.

### See Also the Following Articles

Calcium, Biological Fitness of • Calcium Buffering Proteins: ER Luminal Proteins • Chemiluminescence and Bioluminescence • Phosphatidylinositol-3-Phosphate

### Glossary

**aequorins** Small chemiluminescent proteins that emit blue–green light when they react with calcium ions. They were first isolated from jellyfish and are used to measure and image calcium ions within living cells.

**endoplasmic reticulum (ER)** A network of thin tubes and flat sacs that pervades eukaryotic cells.

**spreading depression** A phenomenon found in the central nervous system that underlies migraine headaches and epileptic seizures (sometimes called Leão's SD). It involves a complex of intense changes that transiently inhibit cell function.

### Further Reading

Fluck, R. A., Miller, A. L., and Jaffe, L. F. (1991). Slow calcium waves accompany cytokinesis in medaka fish eggs. *J. Cell Biol.* **115**, 1259–1265.

Gilkey, J. C., Jaffe, L. F., Ridgway, E. B., and Reynolds, G. T. (1978). A free calcium wave traverses the activating egg of the medaka, *Oryzias latipes*. *J. Cell Biol.* **76**, 448–466.

Jaffe, L. F. (1993). Classes and mechanisms of calcium waves. *Cell Calcium* **14**, 736–745.

Jaffe, L. F. (1999). Organization of early development by calcium patterns. *BioEssays* **21**, 657–677.

Jaffe, L. F. (2002). On the conservation of fast calcium wave speeds. *Cell Calcium* **32**, 217–229.

Jaffe, L.F. (2003). The propagation speeds of calcium action potentials are remarkably invariant. *Biol. Cell (Pans)* **95**, 343–355.

Jaffe, L. F., and Créton, R. (1998). On the conservation of calcium wave speeds. *Cell Calcium* **24**, 1–8.

### Biography

Lionel F. Jaffe is a Senior Scientist at the Marine Biological Laboratory in Woods Hole and at Brown University. His principal research interest is in the broad field of developmental physiology, including calcium waves and calcium gradients, electrical field and polarized light effects, and interactions via chemiluminescent signals. He holds a Ph.D. from the California Institute of Technology.

# Calcium, Biological Fitness of

**Robert J. P. Williams**
*University of Oxford, Oxford, UK*

**Calcium has a special cation, $Ca^{2+}$, in cellular activity. It is rejected by all cell cytoplasm either to the outside of cells or to the inside of resides. From these stores it is released by events outside eukaryote cells to the cytoplasm and organells were it activates many processes. Thus calcium is the dominant messenger, relaying information concerning the environment to activities such as contraction of muscles, release of digestive jucies from the pancreas, to hormone release from glands. It is also the major cation in organizing external protein structures and in formation of biological mineral – shells and bones.**

## Calcium: The Element and its Ion, $Ca^{2+}$

Calcium, atomic number 20 lies in group 2 in the third row of the periodic table and before the first row of transition metals. It resides below magnesium and above strontium and then barium in its group. It has low ionization potentials, giving a rather large calcium ion, $Ca^{2+}$ (radius 1.0Å), which is a rather poor Lewis acid and hence a somewhat poor acid catalyst. However, it can bind quite strongly to certain anion centers. Within its group, the chemistry of the $Ca^{2+}$ ion has close resemblance to that of the larger strontium ion, $Sr^{2+}$, but differs markedly from the smaller magnesium ion, $Mg^{2+}$, which is a better Lewis acid.

Calcium, like magnesium, is an abundant element in the universe and on Earth, and its salts are quite soluble in water. Hence, calcium is available in the sea, $10^{-2}$ M, compared to magnesium at $5 \times 10^{-2}$ M, whereas in fresh water it, like magnesium, is often as high as $10^{-3}$ M. It is against these background levels in water, especially in the sea, that life evolved.

The calcium ion has three major mass isotopes, 40, 42, and 44, which can be used to follow the reactions of its ions. It can also be followed in biological systems through association with fluorescent organic dyestuffs.

### The Aqueous Calcium Ion

The free aqueous calcium ion has a variable fluctuating number of water molecules around it, exceeding six but not exceeding nine. This loose hydration sphere is common to all cations of a radius greater than 0.8Å. In this way the calcium ion differs from the magnesium ion, radius 0.6Å, which has a fixed, rather rigid coordination of six water molecules. The differences in structure are reflected in the rates of exchange of the water molecules from their respective coordination spheres, that of magnesium being rather slow, $10^6\ s^{-1}$ and that of calcium being close to the diffusion limit of $10^9\ s^{-1}$.

### Insoluble Salts

The binding of calcium, a large ion, is less than that of magnesium when the in-coming anion is small (e.g., fluoride or hydroxide) and these magnesium salts tend to be the more insoluble. However, larger groups, which include the vast majority of anions (e.g., carbonate, sulfate, and phosphate anions), are more readily accommodated around the larger calcium ion. Hence, we observe that the vast majority of calcium salts are more insoluble than the corresponding magnesium salts. This becomes a feature in organisms, where we find calcium carbonate in shells and calcium phosphate in bones, whereas in plants calcium oxalate is often precipitated. Aberrant precipitation causes stones in animal organs. Among minerals, calcium and magnesium often occur together, as, for example, in dolomite.

### Soluble Complex Ions and their Binding Strengths

Calcium ions do not bind strongly to simple anions in water at pH 7. Thus, complexes with carbonate, sulfate, hydroxide, and phosphate are not of great importance in organisms. However, binding to ligands with several donor atoms can be strong because despite their bulkiness they can be fitted around the large calcium ion. By contrast, the $Mg^{2+}$ ion fails to bind strongly, so these chelating ligands selectively bind calcium relative to magnesium by at least a thousandfold. Typical examples are proteins both inside cells and in external circulating fluids. The use of the calcium buffer (ethylene tetra-acetate (EGTA)) depends on this quite strong

 © 2004, Elsevier Inc. All Rights Reserved. 

binding to calcium but weak binding to magnesium at pH 7.

### Structures of Calcium Salts and Complexes

The structures of many calcium salts have been determined, and the extremes of their variation are shown even within the allotropic forms of its carbonates. Whereas calcite has a six-coordinate lattice, aragonite has a nine-coordinate structure. These salts have no water of crystallization and both are found in shells. More frequently, when calcium ions are bound to bulky chelating organic ligands in solution, calcium has seven near-neighbors, but the distances from the metal ion to the nearest ligand atom are very irregular, from 2.2 to 3.0Å. Bound to saccharides or proteins, the ion has this structure, but it fluctuates. It is usual for one or two water molecules to remain bound. The binding ligand atoms are invariably oxygen from such centers as oxyanions, carboxylates, alcohols, carbonyls, and ethers.

### Rates of Exchange

The rate of loss of water molecules from around $Ca^{2+}$, $10^9\ s^{-1}$, is the rate at which ligands can bind, $k_{on}$. The rate of leaving is related to the ligand binding constant, $K = k_{on}/k_{off}$, so for $K = 10^6\ M^{-1}$ the leaving rate is $10^3\ s^{-1}$. This binding constant is close to that found for several proteins inside cells. The combination of such a protein (e.g., calmodulin) with calcium then provides a response time of $10^3\ s^{-1}$, as is seen in fast muscle cells. It is the rates of exchange together with the *selective* binding constants that make calcium an incomparable messenger in aqueous solution.

## Biological Salts and Ligand Complexes

The major insoluble calcium salts found in organisms are the fluoride (in krill), carbonates (in shells in at least three allotropic forms), phosphates (in bone), and oxalates (in many plants). There are variable amounts of other metal ions and anions in these salts, so their exact nature is far from simple; for example, bone, hydroxy apatite, does not have an ideal stoichiometric formula and easily incorporates other elements including magnesium and fluoride. The apatite structure is somewhat different in bones of different organisms and even in the teeth and bones of one species (e.g., humans). Calcite is also found in the ears of animals, with a balance function.

In cellular complexes, calcium is bound, seven-coordinate, to oxygen (O-) donor groups of proteins and polysaccharides through both side chain and main chain atoms (e.g., $-OH$, $>CO$, $CO_2^-$, ether, and $SO_4^{2-}$). There is little or no binding to DNA or RNA in cells, in marked contrast to the essential function of magnesium in the structures of these nucleotides. The selective binding arises from the differences in concentration and binding ability.

### Concentrations and Selectivity of Binding in Organisms

The ability of calcium ions to precipitate many organic anions including DNA required that one primary necessity of the original life forms, prokaryotes, was the rejection of calcium. Consequently, all organisms hold $Ca^{2+}$ concentrations at $10^{-6}$ M or below in their cytoplasm using calcium outward pumps. Magnesium ions do not precipitate organic anions and are found at $10^{-3}$ M in all cell cytoplasms. Hence, for $Ca^{2+}$ to bind selectively inside cells, its binding constant must be close to $10^6\ M^{-1}$, whereas that of $Mg^{2+}$ has to be less than $10^3\ M^{-1}$. This selectivity between the two ions is found for many cytoplasmic messenger proteins, for example, calmodulin, troponin and S-100. Meanwhile, $Ca^{2+}$ fails to bind to such molecules as ATP and DNA, $K = 10^4$, but $Mg^{2+}$ can bind with the same binding constant. Outside cells, both $Mg^{2+}$ and $Ca^{2+}$ are $> 10^{-3}$ M and in eukaryotes the cell vesicles hold $Ca^{2+}$ at this concentration also. $Ca^{2+}$ can again bind selectively if its binding constant to proteins or now saccharides is $\geqslant 10^3\ M^{-1}$, whereas that of $Mg^{2+}$ is less than $10^2\ M^{-1}$, because both ions are present outside cells at $10^{-3}$ M. These constants are found for some coat, blood-clotting, and immune-response proteins and for some vesicle proteins. Note that in higher organisms the free $Ca^{2+}$ concentration in external fluids and vesicles is extremely well controlled and in the extracellular fluid of humans is nearly in equilibrium with the precipitated bone. The further requirement for a messenger activity, that the rate of exchange to the internal proteins be fast, at least $10^3\ s^{-1}$ for calcium, is also met.

Consider now a cytoplasmic concentration in a resting cell of $Ca^{2+}$ of $10^{-7}$ M and an external or vesicle concentration of $10^{-3}$ M. Any influx of $Ca^{2+}$ to the cytoplasm from outside through channels will increase $Ca^{2+}$ to $10^{-6}$ M, which will trigger activity through cytoplasmic protein binding. On closing the channel, the outward pumps will restore rest conditions quickly. The calcium ion is therefore able to act as a pulsed messenger. Hence, the combination of sufficiency of binding, of selectivity, of concentration control, and of rates of on/off reactions give optimal messenger character to this elementary ion. No other ion or molecule could be better.

## Overall Functional Fitness of Calcium Ions in Evolution

The properties of the calcium ion, its fast on/off reactions reflecting the structural flexibility of its coordination sphere and its quite strong binding to selected organic ligands, have made it an extremely valuable partner to organic chemicals in almost all forms of life. In the most primitive forms of life, this value is seen in protective external constructs (walls) and mineralization, but also in the use of bound calcium in external digestive catalytic enzymes. These functions are found in all other organisms but the most striking feature of the use of calcium appeared at the time the eukaryotes developed from the prokaryotes. At that time, the problem of coordinating activities of different compartments (vesicles) and the need for an increasing recognition of the environment became critical for the survival of the large eukaryote cells. Cooperation between compartments and sensing required the rapid transfer of information between cellular compartments and from the outside environment, and there is no better possible mode of communication in cellular biology than that which can be derived from the happy coincidental values of the high availability with concentration control and the response properties of the calcium ion. Hence, it was an evolutionary requirement that led to a messenger system in which the calcium ion was the best of all available carriers (Table I). The protein receptors in the cytoplasm acted as mechanical triggers of action, changing conformation upon binding calcium. They include all muscle responses (troponins), fertilizations, many metabolic responses (calmodulins), genetic activations (possibly S-100 and other proteins), and immune responses (calcineurin). The necessary refinement of the homeostasis of the calcium concentrations increased all the way up to humans and included the control over both the internal cytoplasmic and the external circulating fluid concentrations. In the external fluids, there arose various novel functions that used the calcium ion, including further immune responses and blood-clotting controls in addition to digestive actions. Finally, we must remember that the messenger system from outside to inside the cell

**TABLE I**

**Calcium-Controlled Events in Cells**

| Activity | Controlled events or systems |
|---|---|
| Photosynthesis | Dioxygen release |
| Oxidative phosphorylation | Dehydrogenases |
| Receptor responses | Nerve synapse; $IP_3$-linked reactions |
| Contractile devices | Muscle triggering (actomyosin); cell filament controls (tubulins) |
| Phosphorylation | Activation of kinases, e.g., in fertilization |
| Metabolism | Numerous enzymes inside cells |
| Membrane/filament organization | Annexin-like proteins modulate tension |
| Cell division | S-100 proteins, immune system |
| Cell death (apotosis) | Internal proteases |
| Hormone/transmitter release | Homeostasis |
| Binding to membranes | C2 domains of enzymes |
| Cross-linking | Outside cells |
| Enzyme-activation | Outside cells; in membranes |

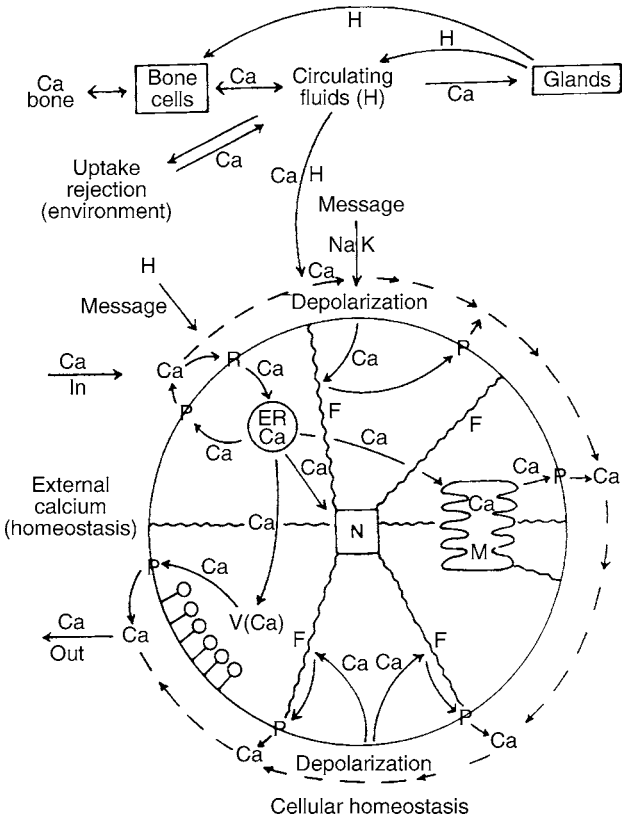



**FIGURE 1** The generalized way in which calcium ions act as a messenger in advanced animal cells. The top of the figure shows the connection between the calcium in the external fluids, bone, and glands and uptake and rejection systems (e.g., the epithelial cells of digestion and the rejection cells of the kidney). H is a hormone. Calcium ions from the circulating fluids together with hormones and Na/K nerve messages activate a large range of cells. The calcium entry is controlled by channels R responding to perturbations. On entering the cell, calcium can release further calcium from vesicles such as the endoplasmic reticulum (ER). The calcium in the cytoplasm can act directly on filaments (F), on mitochondria (M) (or chloroplasts), through transcription factors on the nucleus (N), and on vesicles (V). The vesicles act to release transmitters, hormones, or enzymes. The calcium is removed by pumps (P). The bottom of the diagram shows the effect of general depolarization rather than specific channel activation. This allows calcium entry by electrically operated channels, as, for example, at nerve synapses of the heart.

was augmented by the further responses, releases due to calcium pulses of chemicals, which caused release from internal vesicles and organelles to both the cytoplasm and the external fluids. Thus, the pulsed calcium ion input to the mitochondria and chloroplasts stimulates the release of energy (ATP) to the cytoplasm, and the release of this ion from vesicles to the external fluids stimulates the release not only of enzymes (e.g., for digestion, general to many organisms) but also of hormones and synaptic messengers, leading to overall homeostatic control. Hence, calcium signaling is central to a huge variety of communication modes leading up to and including synaptic action in the brain. In this homeostasis, we must not forget the buffer action of the bone. The overall fitness of calcium for a multitude of functions is very clear (see Figure 1).

## See Also the Following Articles

Calcium Oscillations • Calcium Signaling: Calmodulin-Dependent Phosphatase • Calcium Signaling: Cell Cycle • Calcium Transport in Mitochondria • Calcium Waves • ER/SR Calcium Pump: Function • ER/SR Calcium Pump: Structure • Ligand-Operated Membrane Channels: Calcium (Glutamate) • Plasma Membrane Calcium Pump: Structure and Function • Store-Operated Membrane Channels: Calcium

## Glossary

**biological minerals** Shells and bones.
**calcium complexes** Soluble combinations of calcium with ligands.
**calcium ion properties** Ion size, ionization potential, and fast exchange.
**fitness** The Darwinian concept of being valuable for a biological function.
**messenger function of calcium** Calcium moving between internal compartments of a cell or between external and internal zones.
**organelles** Mitochondria or chloroplasts. They differ from vesicles in having residual DNA/RNA synthetic systems, although both organelles and vesicles are compartments separated by membranes from the cytoplasm.

## Further Reading

Carafoli, E., and Klee, C. (eds.) (1999). *Calcium as a Cellular Regulator.* Oxford University Press, New York.
Carafoli, E., and Krebs, J. (eds.) (2000). *Calcium Homeostasis*. Springer Verlag, Berlin.
Fraústo da Silva, J. J. R., and Williams, R. J. P. (2001). Chapters 10 and 20. *The Biological Chemistry of the Elements*. Oxford University Press, Oxford.
Pochet, R. (ed.) (2000). *Calcium: The Molecular Basis of Calcium Action in Biology and Medicine*. Kluwer Academic Press, Dordrecht, The Netherlands.
Vogel, H. J. (ed.) (2002). *Calcium-Binding Protocols*, Vols. 1–2, Humana Press, Totowa, N.J.

## Biography

Robert J. P. Williams is Emeritus Professor at Oxford University, having been a Royal Society Research Professor there for many years. He holds an M.A., D.Phil. (Oxford), F.R.S. (London), Hon. D.Sc. (East Anglia, Leicester, Keele) and is a Fellow of the Academies of the Czech Republic, Belgium, Sweden and Portugal. His research interests are in bio-energetics and the roles of inorganic ions in organisms. He has received many honors: four honorary doctorates and more than ten medals from chemical and biochemical societies. He is an academy member of four national bodies. His founding contribution was to biological inorganic chemistry including the development of the protein chemistries of elements such as Ca, Fe, Cu, Zn, Co, and Mg; he also proposed the use of protons as intermediates in bio-energetics. He is the author of four leading books in these areas.

# Calcium/Calmodulin-Dependent Protein Kinase II

**Andy Hudmon**
*Yale University, New Haven, Connecticut, USA*

**Howard Schulman**
*Mountain View, California, USA*

**Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine (Ser/Thr) protein kinase involved with a diverse array of cellular functions. From metabolism and cytoskeletal organization to gene expression and the cell cycle, CaMKII phosphorylation of key substrates transduces a transient rise in free calcium ($Ca^{2+}$) induced by neurotransmitters, hormones, or other signaling molecules, into complex cellular functions. CaMKII is particularly important in the nervous system, where neurogenetics (knockouts, knockins, and transgenics) have directly implicated it in behavior and learning/memory. It is strategically localized in both pre- and postsynaptic compartments, where it regulates $Ca^{2+}$-dependent processes central to neuronal communication, including neurotransmitter synthesis and release and ion flux through voltage and ligand-gated channels. In addition, CaMKII possesses autoregulatory properties that may enable it to function as a "molecular switch" to translate neuronal activity into specific alterations in synaptic connectivity, such as long-term potentiation (LTP), a cellular correlate of learning and memory. Autophosphorylation can be considered as a form of molecular memory, as it generates a form of persistent activity (autonomous) that is temporally uncoupled from the $Ca^{2+}$ signal. This autoregulation also enables CaMKII to function as a $Ca^{2+}$ spike integrator – the ability to generate graded levels of autonomous activity that is differentially sensitive to the frequency of $Ca^{2+}$ transients. Thus, its direct action on neuronal substrates and its complex autoregulation enables CaMKII to function as a cognitive kinase with both short- and long-term consequences on complex behaviors, such as learning and memory.**

## Neuronal CaMKII: Localization and Substrates

CaMKII is highly concentrated in the nervous system. At roughly 1–2% of the total protein in the brain, it is the predominant $Ca^{2+}$/calmodulin ($Ca^{2+}$/CaM) regulated kinase in the central nervous system. CaMKII has a diverse intracellular localization, including the postsynaptic density (PSD), a cytoskeletal specialization replete with signaling molecules (e.g., ligand and voltage gated receptors, kinases, phosphatases, and anchoring and structural proteins) that lies immediately across the presynaptic terminal below the postsynaptic membrane. Biochemical cascades at the PSD regulated by CaMKII are likely central to the function of this highly specialized signaling network for the following reasons: (1) CaMKII constitutes a significant fraction of the total PSD protein, (2) it is recruited to the PSD in an activity-dependent manner, (3) it anchors to multiple PSD proteins (e.g., densin-180, $\alpha$-actinin, and NMDA-receptors), and (4) it regulates the activity of multiple PSD proteins through phosphorylation. One prominent PSD substrate of CaMKII is the GluR1 subunit of the AMPA-subtype of glutamate receptor. CaMKII phosphorylation of a specific serine ($Ser^{831}$) in this channel increases its conductance by favoring the open probability of its high conductance state. CaMKII was also shown by Malinow and his colleagues to regulate the targeting or insertion of AMPA-receptors into the PSD. Although both effects may underlie long-term potentiation, CaMKII regulation of AMPA receptor function and targeting has obvious implications for postsynaptic function; further studies will be required to determine how the 28 CaMKII substrates in the PSD recently identified using mass spectrometry contribute to PSD structure and function.

In addition to the postsynaptic compartment of neurons, CaMKII is also present in the presynaptic compartment, where it regulates neurotransmitter synthesis, neurotransmitter vesicle availability and release by its phosphorylation of vesicle proteins (e.g., Synapsin I) cytoskeletal regulating proteins (e.g., MAP2), membrane channels (e.g., calcium channels), and other enzymes (e.g., Tyrosine Hydroxlyase). CaMKII is also present in the nucleus, where it phosphorylates key

 © 2004, Elsevier Inc. All Rights Reserved. 

substrates (e.g., CREB) involved in the regulation of gene transcription and long-term changes in neuronal function. Thus, CaMKII is strategically positioned throughout the neuron to transduce and coordinate a host of calcium mobilizing stimuli into the appropriate alterations in neuronal function (see Figure 1).

## Activation, Autoregulation, and Structure of CaMKII

CaMKII is composed of a family of highly conserved isoforms ($\alpha$, $\beta$, $\gamma$ and $\delta$ that are encoded by unique genes and differentially expressed throughout the body (see Figure 1). Although the $\delta$ and $\gamma$ isoforms are ubiqitous, the $\alpha$ and $\beta$ isoforms are brain specific. The molecular weights of these subunits range from 54 to 72 kDa, with $\alpha$-isoform being the smallest subunit at 54 kDa. This size difference is due primarily to domain insertions in the region between the regulatory and association domains, because each of these genes encode protein products possessing a catalytic, regulatory, and association domain. A proto-typical $\alpha$-subunit of CaMKII, illustrating the relative positions of these domains, is shown in Figure 2. Like all protein kinases, the catalytic domain serves to bind substrates and catalyze the phosphotransferase reaction from $Mg^{2+}$/ATP to the protein substrate.

CaMKII is coupled to the $Ca^{2+}$ stimulus via CaM, a small dumbbell-shaped protein that, following $Ca^{2+}$ binding, is competent to bind and activate multiple target proteins, including CaMKII. The autoregulatory domain is composed of a CaM binding domain and a region that resembles a protein substrate. This pseudo-substrate and the surrounding sequence of the regulatory domain (including the CaM binding domain) interacts with the catalytic domain to inhibit kinase activity in the absence of $Ca^{2+}$/CaM. C-terminal to the autoregulatory domain, the association domain produces the supramolecular association of kinase subunits into a multimeric complex, termed the holoenzyme.

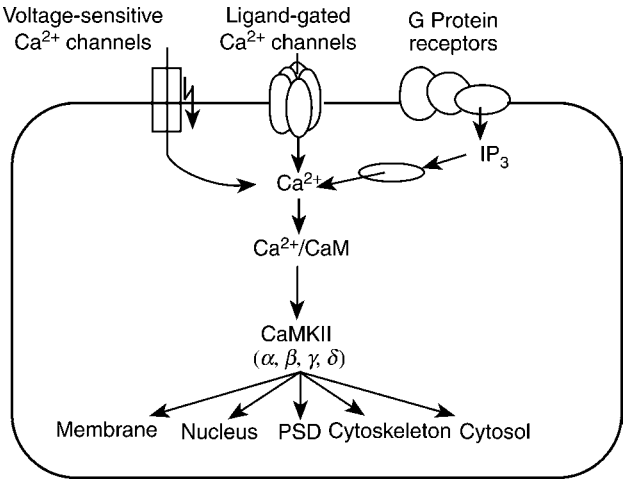



**FIGURE 1** A variety of extracellular signals (e.g., depolarization, neurotransmitters, and hormones) function to elevate intracelular $Ca^{2+}$ levels through the action of ligand and voltage-gated channels and $IP_3$ operated intracellular stores. Activation of CaMKII ($\alpha$, $\beta$, $\gamma$, and $\delta$ isoforms) throughout the neuron via $Ca^{2+}$/CaM leads to phosphorylation of substrates at the membrane, cytoskeleton, postsynaptic density (PSD), nucleus, and in the cytosol. Adapted with permission from *Annu. Rev. Biochem.* **71**, 473–510 by Annual Reviews.

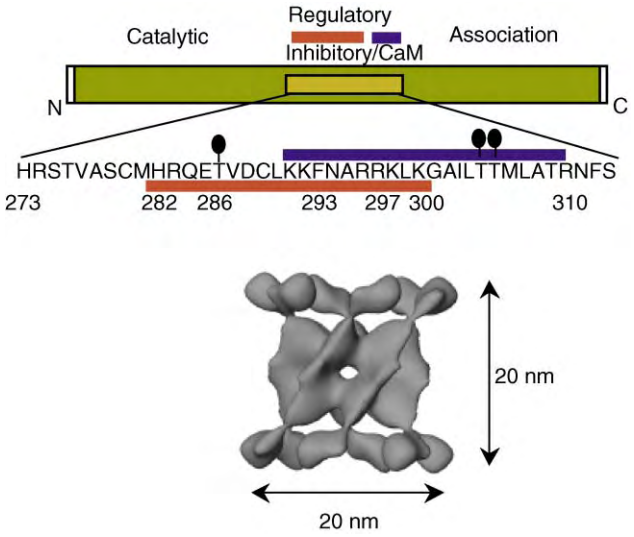



**FIGURE 2** Linear diagram of $\alpha$-CaMKII illustrating the catalytic, regulatory, and association domains. Like all kinases, the catalytic domain is composed of both an ATP and a protein substrate binding region. Adjacent to the catalytic domain is an autoregulatory region (shown in yellow): a bifunctional sequence that serves to inhibit kinase activity through an inhibitory region (red) and to activate the kinase via the calmodulin binding domain (blue). The association domain drives subunit multimerization and holoenzyme assembly. A computer assisted reconstruction of $\alpha$-CaMKII obtained from images produced using transmission electron microscopy indicates a dodecameric structure, with 12 catalytic heads radiating out form the top and bottom of the central core.

A computer-assisted reconstruction of $\alpha$-CaMKII obtained from images using transmission electron microscopy indicated that the catalytic heads extend from the gear-shaped central core via delicate stalk in a dodecameric (12 subunits) arrangement (Figure 2). The structure of the core hub-like assembly was recently resolved in a crystal obtained from a truncated form of CaMKII. In this structure, the association domain appeared as a tetradecameric structure (14 subunits) with extensive contacts between neighboring subunits in a head-to-head orientation. Variance in the number of subunits forming the holoenzyme may be expected, as native CaMKII isolated from forebrain varies greatly in its apparent molecular weight (450–650 kDa). In addition, CaMKII isolated from brain may be homo- and heteromeric in its isoform composition.

The isoform composition of the holoenzyme may afford CaMKII with the properties and regulation unique to each isoform. For example, in addition to the $\alpha$ and $\beta$ isoforms being differentially expressed throughout the brain and during development, recent evidence indicates that neuronal activity favors the expression of the $\alpha$-isoform whereas, a lack of activity favors the $\beta$-isoform. One functional consequence of altering the subunit composition is in its regulation of the subcellular localization of CaMKII. For example, unlike the $\alpha$-isoform, the $\beta$-isoform possesses an F-actin binding domain that targets this isoform to cytoskeletal structures, where it participates in regulating the sprouting and stability of neuronal dendrites.

## The Structure/Function of CaMKII Autophosphorylation

In addition to phosphorylating protein substrates throughout the cell (see Figure 1), CaMKII is a substrate for its own catalytic activity via *auto*phosphorylation reactions. CaMKII undergoes both $Ca^{2+}$/CaM-dependent as well as $Ca^{2+}$/CaM-independent forms of autophosphorylation. These modes of autophosphorylation occur at distinct sites within different regions of the autoregulatory domain and, importantly, have both been implicated in both the cellular correlates of learning and memory (i.e., LTP), as well as the behavior itself.

### $Ca^{2+}$/CaM-Dependent Autophosphorylation

Following CaM activation, autophosphorylation may occur at $Thr^{286}$ ($Thr^{287}$ of the $\beta$, $\gamma$ and $\delta$), a phosphoamino acceptor that resides just N-terminal to the pseudo substrate anchor and CaM binding domain (see Figure 2). Autophosphorylation at $Thr^{286}$ is not necessary for enzyme activation, because nonphosphorylatable amino acids (e.g., Ala) substituted at position 286 are similar to wild-type enzyme in regards to both CaM activation and substrate phosphorylation. The autophosphorylation reaction is dominated by an intraholenzyme inter-subunit reaction that requires CaM bound to both the subunit acting as kinase as well as the subunit behaving as substrate (Figure 3). Three functional consequences associated with $Thr^{286}$ autophosphorylation are (a) the generation of

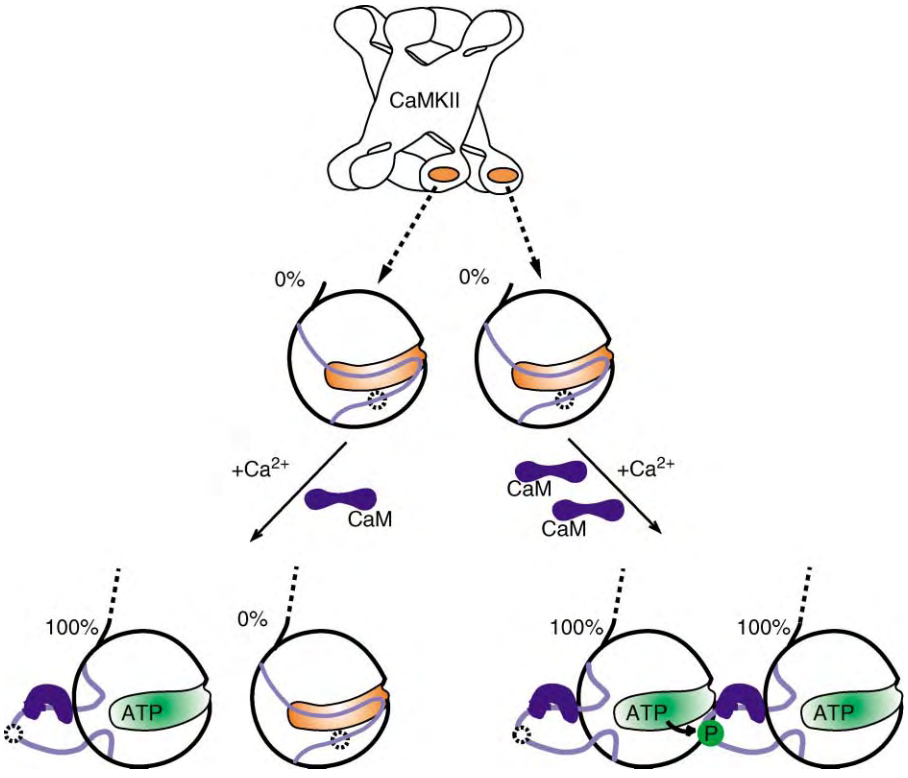



**FIGURE 3** Autophosphorylation requires coincident $Ca^{2+}$/CaM binding between neighboring subunits within CaMKII. Catalytic/regulatory regions are depicted from subunits within the same holoenzyme (i.e., intraholoenzyme) and CaM is depicted as the purple dumbbell. $Ca^{2+}$/CaM binding displaces the autoinhibitory domain to activate subunits within the holoenzyme. Coincident binding of $Ca^{2+}$/CaM exposes the autonomy site ($Thr^{286}$), represented as a dashed circle, of one subunit for phosphorylation by an active neighboring subunit in an intersubunit reaction. Adapted with permission from *Annu. Rev. Biochem.* **71**, 473–510 by Annual Reviews.

autonomous activity, (b) an increase in the binding affinity of CaM (CaM trapping) and (c) exposure of a site on the catalytic domain that functions as a binding site to anchor CaMKII to its substrates.

### Autonomous Activity

Autonomous activity ($Ca^{2+}$/CaM-independent activity) has intrigued neurobiologists and enzymologists since its discovery almost 20 years ago in the marine mollusc, *Aplysia californica* – a well-characterized animal model for studying the molecular mechanisms of learning and memory. Autonomous activity can be described as the "residual" activity attributed to an autophosphorylated subunit after CaM dissociation. At the structural level, the mechanism of autonomous activity is likely attributed to the negative charges associated with phospho-$Thr^{286}$ preventing reassociation and inactivation of the catalytic domain by the autoregulatory domain following CaM dissociation.

### CaM Trapping

Among the various CaM target proteins reported, CaMKII possesses a relatively low binding affinity for CaM ($K_d$ 15 nM). However, following autophosphorylation, the CaM binding affinity is decreased by over a 1000-fold ($K_d$ 20 pM), thus attaining one of the highest CaM binding affinities reported to date. The autophosphorylation of $Thr^{286}$ is thought to indirectly produce CaM trapping, because a peptide designed from the $Ca^{2+}$/CaM binding domain of CaMKII (290–309) possesses high affinity $Ca^{2+}$/CaM binding, even though $Thr^{286}$ is not contained within the peptide sequence (see Figure 2). This is mechanistically consistent with steric constraints or an inaccessibility of the binding site for $Ca^{2+}$/CaM in the nonautophosphorylated state. In support of the peptide binding studies, mutant cycle analysis studies designed to delineate the amino acid contacts responsible for high affinity binding between CaM and CaMKII supports a model for CaM trapping whereby $Thr^{286}$ autophosphorylation exposes amino acids ($Phe^{293}$ and $Arg^{294}$) within the N-terminal part of the CaM target sequence to thermodynamically stabilize and expose the target sequence for optimal $Ca^{2+}$/CaM binding.

### Substrate Anchoring

The subcellular targeting of multifunctional kinases (e.g., CaMKII, cAMP-dependent protein kinase, and protein kinase C), is thought to be an integral component in generating the kinetics of the phosphorylation reaction and substrate specificity. $Ca^{2+}$/CaM binding and autophosphorylation of $Thr^{286}$ exposes a binding site on CaMKII to permit anchor proteins and substrates to directly associate with the enzyme. Protein targeting may also afford new modes of regulation for CaMKII. For example, CaMKII targeting to the NR2B subunit of the NMDA-type glutamate receptor requires $Ca^{2+}$/CaM to induce binding. However, once the interaction is formed, the enzyme is active and remains bound *even* in the absence of $Ca^{2+}$/CaM and $Thr^{286}$ autophosphorylation. Binding of the NR2B subunit to the catalytic domain may act as a wedge to prevent the autoregulatory domain from reforming its contacts subsequent to CaM dissociation.

## Autophosphorylation at $Thr^{305}$/$Thr^{306}$

$Ca^{2+}$/CaM-independent autophosphorylation occurs at amino acids $Thr^{305}$/$Thr^{306}$ with the CaM binding domain. Often referred to as inhibitory or "burst" autophosphorylation, $Ca^{2+}$/CaM-independent autophosphorylation is initiated once $Ca^{2+}$/CaM dissociates from a kinase phosphorylated at $Thr^{286}$. Autophosphorylation within the CaM binding domain ($Thr^{305}$ or $Thr^{306}$) disrupts CaM from rebinding to this subunit. Autophosphorylation in the $Ca^{2+}$/CaM binding domain has also been reported in the basal state (autoinhibited), a process that appears to occur by an intrasubunit mechanism and is likely a result of this regions proximity to the substrate binding pocket on the catalytic domain. Basal autophosphorylation preferentially occurs at $Thr^{306}$, and like burst autophosphorylation, it functions to block $Ca^{2+}$/CaM binding.

## Autophosphorylation as an Index of Neuronal Activity

In neurons, like in other excitable cells, $Ca^{2+}$ signaling is a balance between homeostatic mechanisms (e.g., $Ca^{2+}$ binding proteins and membrane extrusion pumps) designed to maintain and buffer the intracellular free $Ca^{2+}$ concentration at roughly 20,000-fold below extracellular levels and a transient rise in free $Ca^{2+}$ induced by hormones, neurotransmitters, or other signaling molecules. Autonomous activity is a form of persistent activity that is temporarily uncoupled from $Ca^{2+}$ signaling. In addition to possessing the capacity to potentiate its activity beyond the $Ca^{2+}$ transient, CaMKII is also capable of translating the temporal dynamics (e.g., frequency) of $Ca^{2+}$ signaling. Immobilized CaMKII subjected to $Ca^{2+}$/CaM pulsed at varying frequencies by a high pressure computer controlled valve assembly generates distinct graded levels of autonomous activity *in vitro*. Two factors identified in computer simulations as key for frequency decoding are the effective cooperativity in the $Thr^{286}$ autophosphorylation reaction and the availability of CaM for this reaction. In cells, CaM is thought to be largely bound to a variety of cellular proteins and presumably not available to maximally activate all of its targets. CaMKII

activation and autophosphorylation are particularly sensitive to limiting CaM levels as its relatively high affinity for CaM would predict that a transient rise in $Ca^{2+}$ would lead to only a few of the subunits in each holoenzyme being activated (Figure 4). Under these conditions, a minority of activated subunits would actually undergo $Thr^{286}$ autophosphorylation and trap CaM due to the requirement of coincident CaM binding for the autophosphorylation reaction. Higher $Ca^{2+}$ spike frequencies produce shorter $Ca^{2+}$ spike intervals that increase the CaM remaining associated between $Ca^{2+}$ transients and the probability of autophosphorylation. Because autophosphorylation further prolongs CaM association, a feed-forward process ensues at a threshold level of autophosphorylation to produce the nonlinear $Ca^{2+}$ spike frequency function for CaMKII activation/autophosphorylation illustrated in Figure 4. The frequency response function of CaMKII could also be influenced by autophosphorylation within the CaM binding domain, because inhibitory autophosphorylation could function to limit activated/autonomous subunits from subsequently competing with nonactivated subunits for the available free CaM.

## CaMKII in LTP and Learning and Memory

Neuronal information in the brain is propagated and encoded through synaptic activity and multiple lines of evidence indicate that CaMKII is necessary and sufficient to induce long-lasting changes in synaptic efficacy, such as long-term potentiation (LTP). LTP is a long-lasting enhancement in the strength of neurotransmission that is believed to represent a cellular correlate of learning. Synaptic stimulation protocols that produce LTP in the hippocampus also produce long-lasting changes in the activation and autophosphorylation of CaMKII. Importantly, peptide inhibitors of CaMKII are potent inhibitors of LTP induction and viral introduction of a

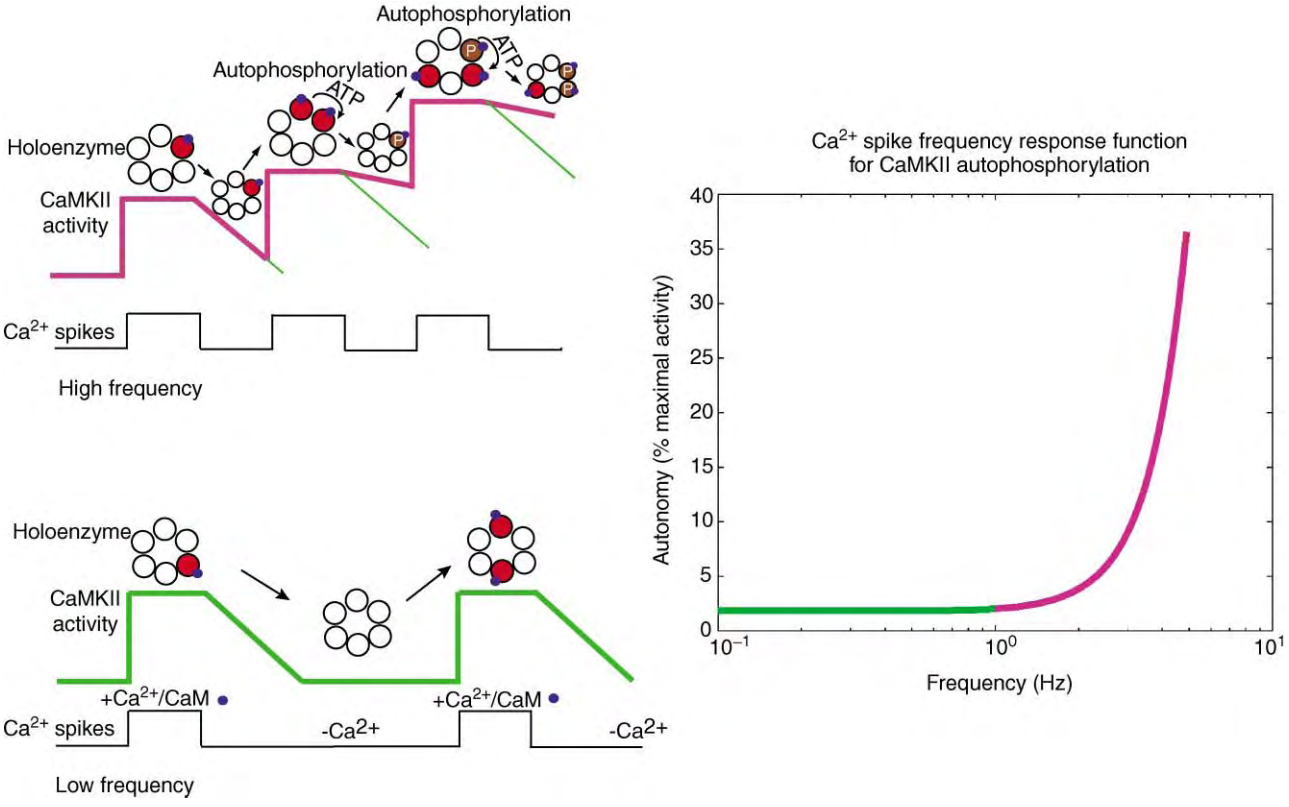



**FIGURE 4** (Left) The role of coincident CaM binding, autophosphorylation, and CaM trapping in $Ca^{2+}$ spike frequency detection. Inactive subunits within CaMKII (holoenzymes are shown as 6 mers for simplicity) are represented by open circles. $Ca^{2+}$/CaM (blue circle) binding activates a given subunit (shown in red) and coincident $Ca^{2+}$/CaM binding results in autophosphorylation and CaM trapping ("P" inside a filled dark red circle). During low frequency $Ca^{2+}$ spikes, CaM completely dissociates between $Ca^{2+}$ spikes, effectively producing "naïve" CaMKII subunits at each inner spike interval. However, at high $Ca^{2+}$ spike frequencies, CaM does not completely dissociate between $Ca^{2+}$ spikes; this increases the probability that coincident CaM binding, autophosphorylation, and CaM trapping may occur – a process that effectively increases the probability that a neighboring subunit will also bind $Ca^{2+}$/CaM during successive spikes to produce the nonlinear increase in autonomous activity illustrated by a computer simulation encompassing the biophysical and enzymatic characteristics of CaMKII (Right). Adapted with permission from *Biochem. J.* **364**, 593–611 © the Biochemical Society.

constitutively active form of CaMKII occludes LTP generated by the high frequency stimulation protocols typically used to induce LTP. In addition to being implicated in synaptic plasticity, the activity and autophosphorylation of CaMKII are also important for learning and memory.

In the field of neuroscience as in other areas of biology, genetic approaches in flies, worms, and mice have greatly advanced our understanding of the molecular mechanisms involved in behavior and learning. Although we will focus on the role of CaMKII in mammalian learning, disrupting the activity of CaMKII induces behavioral or cognitive defects in *all* of these organisms. The genetic deletion in the $\alpha$-isoform of CaMKII was a pioneering study aimed at understanding the role of CaMKII in mammalian learning and memory. These CaMKII knockout mice displayed deficits in hippocampal LTP and possessed severe learning impairments in spatial learning tasks, such as the Morris Water Maze. These knockout mice directly implicated CaMKII and hippocampal LTP in mammalian learning and memory.

The use of knockin genetics permitted researchers to introduce mutant forms of CaMKII into both wild-type and knockout mice. This technology permitting further dissection of the role of CaMKII in learning, as mutant mice could be created that had altered autoregulatory properties without ablating kinase activity. A knockin mouse in which $Thr^{286}$ ($\alpha$-isoform) was mutated to $Ala^{286}$ to prevent autophosphorylation displayed deficient LTP and spatial learning, directly implicating $Thr^{286}$ autophosphorylation in synaptic plasticity and learning. Recent knockin technology has also permitted temporal control of transgene expression with inducible promoters. In experiments with a transgenic mouse in which $Thr^{286}$ was replaced with a charged residue (e.g., $Asp^{286}$) in order to mimic an autophosphorylated state of the enzyme, enhanced LTP at low levels of transgene expression and deficient LTP at higher levels occurred, suggesting complex interactions between the level of activated CaMKII and its function in regulating synaptic strength. Autophosphorylation within the CaM-binding domain ($Thr^{305}$/$Thr^{306}$) has also been implicated in regulating CaMKII targeting and function. Knockin mice that cannot undergo inhibitory autophosphorylation ($Ala^{305}$/$Ala^{306}$) have increased levels of CaMKII in the PSD and a lower threshold for LTP. Mimicking inhibitory autophosphorylation ($Asp^{305}$/$Asp^{306}$) lowers the CaMKII content in the PSD and blocks LTP induction and hippocampal learning. Finally, in addition to CaMKII autophosphoryation regulating synaptic plasticity and behavior, a knockin mouse with an intact coding region for $\alpha$-CaMKII yet possessing an altered mRNA targeting was created. Disrupting mRNA localization into dendrites was found to impair LTP and learning, suggesting that local translation and delivery of CaMKII to its site of action at the synapse is important for synaptic plasticity learning.

## The Tao of Neuronal CaMKII

The function of CaMKII in synaptic plasticity and learning and memory appears to be tightly linked to its unique structure and autoregulation. The multimeric structure of CaMKII permits different isoforms to associate into a macromolecular signaling complex to provide specific targeting information essential for its subcellular localization as well as an intersubunit autophosphorylation reaction. Key autoregulatory features following autophosphorylation include the ability to (1) reduce its $Ca^{2+}$/CaM dependence following dissociation of CaM (autonomous activity), (2) increase its affinity for $Ca^{2+}$/CaM by over 1000-fold (CaM trapping), (3) enable activity-dependent substrate targeting, and (4) enable it to function as a $Ca^{2+}$ spike frequency detector. Thus, the designation of a kinase as a cognitive kinase (i.e., based on its requirement in learning and memory) possesses a double meaning for CaMKII. Because of this enzyme's complex autoregulation and autophosphorylation, CaMKII activity may directly function as a molecular representation of synaptic activity: a molecular memory that is expressed as a persistent form of activity temporarily uncoupled from the $Ca^{2+}$ stimulus. Thus, the role of CaMKII in transducing synaptic activity into the complex neuronal changes associated with learning appears to be an intimate dance between its autoregulation and its capacity to phosphorylate key protein substrates integral to neuronal function.

### See Also the Following Articles

Protein Kinase C Family • Voltage-Sensitive $Ca^{2+}$ Channels

### Glossary

**$Ca^{2+}$ transient** A temporary rise in the intracellular levels of free $Ca^{2+}$ ($\approx$100 nM to 1 $\mu$M) induced by a variety of extracellular signals (hormones, neurotransmitters, and depolarization) that may vary in its localization, duration, frequency, and amplitude.

**hippocampus** Part of the limbic system of the brain that is involved in spatial learning; because of its laminar architecture, it is routinely used for electrophysiological studies designed to evaluate synaptic connectivity and plasticity.

**long-term potentiation** A long-lasting increase in synaptic strength that could function to strengthen synaptic connectivity; it is considered to represent a cellular correlate of learning and memory.

**Morris water maze** An apparatus that is routinely used to assess spatial learning and memory in rodents. In this task, a mouse is placed in a large tub of an opaque liquid and assayed both for its ability to find a visible platform as well as a submerged

platform – tasks that evaluate the general function and ability to learn the task as well as its spatial learning, respectively.

**pre- and postsynaptic elements of neurons** Components of typical neuronal synapse: the presynaptic terminal releases neurotransmitter that diffuses across a synaptic cleft to bind and activate ligand-gated channels along the postsynaptic specialization.

**pseudosubstrate** A component of the autoregulatory domain that binds to the substrate binding pocket on the catalytic domain to prevent kinase activity in the absence of calmodulin.

## Further Reading

Griffith, L. C. (1997). Drosophila melanogastor as a model system for the study of the function of calcium/calmodulin-dependent protein kinase II in synaptic plasticity. *Invert. Neurosci.* **3**, 93–103.

Hudmon, A., and Schulman, H. (2002). Neuronal $Ca^{2+}$/calmodulin-dependent protein kinase II: The role of structure and autoregulation in cellular function. *Annu. Rev. Biochem.* **71**, 473–510.

Kennedy, M. B. (2000). Signal-processing machines at the postsynaptic density. *Science* **290**, 243–257.

Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioral memory. *Nature Rev. Neurosci.* **3**, 175–190.

Miller, S., Yasuda, M., Coats, J. K., Jones, Y., Martone, M. E., and Mayford, M. (2002). Disruption of dendritic translation of CaMKII$\alpha$ impairs stabilization of synaptic plasticity and memory consolidation. *Neuron* **36**, 507–519.

Shen, K., Teruel, M. N., Connor, J. H., Shenolikar, S., and Meyer, T. (2000). *Molecular memory by reversible translocation of calcium/calmodulin-dependent protein kinase II. Nature* **3**, 881–886.

Silva, A. J. (2003). Molecular and cellular cognitive studies of the role of synaptic plasticity in learning and memory. *J. Neurobiol.* **54**(1), 224–327.

Soderling, T. R. (2000). CaM-Kinases: Modulators of synaptic plasticity. *Curr. Opin. Neurobiol.* **10**, 375–380.

## Biography

Andy Hudmon is currently a research scientist at the PVA-EPVA Center for Neuroscience and Regeneration Research in the Department of Neurology at Yale University where he is studying the calcium-dependent regulation of voltage gated sodium channels in models of pain and spinal cord trauma. As a postdoctoral fellow in the laboratory of Dr. Howard Schulman at Stanford University, he studied the targeting and function of the multi-functional $Ca^{2+}$-calmodulin-dependent protein kinase II in regulating voltage-gated calcium channels in the heart and nervous system. He received his M.S. in physiology at Auburn University in the laboratory of Dr. Jim Sartin, and he holds a Ph.D. from the University of Texas Health Science Center at Houston, where he trained in the laboratory of Dr. Neal Waxham.

Howard Schulman is a Professor in the Department of Neurobiology at Stanford University and currently is Vice President of research at Surromed (MountainView, CA). His principle research interest is in how the structure/regulation of protein kinases decode and translate the calcium signal during neuronal function and plasticity. He holds a Ph.D from Harvard University, where he trained under Dr. Eugene Kennedy. Dr. Schulman received his postdoctoral training in the laboratory of Dr. Paul Greengard at Yale University.

# Calcium/Calmodulin-Dependent Protein Kinases

**J. Robison and Roger J. Colbran**
*Vanderbilt University, Nashville, Tennessee, USA*

**The $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) enzyme family is activated by increases in intracellular calcium ions ($Ca^{2+}$) and transfers phosphates from ATP to specific serine or threonine residues in other proteins. Family members vary in expression level, subcellular location, and substrate specificity. They are essential for signal transduction in all cells and modulate a variety of physiological processes including learning and memory, metabolism, gene transcription, and motor function.**

## $Ca^{2+}$ Signaling

Modulation of the concentration of $Ca^{2+}$ is used as an intracellular signal to allow hormones and neurotransmitters to control numerous processes in virtually all cell types. In general, cells have a resting $Ca^{2+}$ concentration of 50–100 n*M*, but this concentration can "spike" above 1 μ*M* in an activated cell. Interestingly, these changes in $Ca^{2+}$ concentration can activate extremely disparate signaling cascades not only in different cell types but also in different areas of the same cell. For instance, $Ca^{2+}$ elevation in the nucleus of a neuron activates gene transcription, whereas a similar elevation at the synapse causes neurotransmitter release. These responses are possible because of the hundreds of different molecules that are directly or indirectly responsive to $Ca^{2+}$, the numerous mechanisms for increasing $Ca^{2+}$, and the variation in duration, frequency, amplitude, and localization of $Ca^{2+}$ spikes. $Ca^{2+}$ can enter the cell through channels in the membrane or be released from internal stores. The resulting $Ca^{2+}$ spikes can occur on the millisecond level in neurons or be separated by days in the mitotic cell and modulate diverse enzymes and/or alterations in the shape of structural molecules. Many of the intracellular effects of $Ca^{2+}$ are mediated by calmodulin, a small protein that undergoes dramatic conformational changes when bound to $Ca^{2+}$ ions. Among the many enzymes activated by $Ca^{2+}$/calmodulin (Figure 1), members of the CaMK family mediate diverse physiological responses in most, if not all, cell types.

Protein kinases are enzymes that catalyze the addition of phosphates onto specific residues in target proteins, thereby regulating them in some way. Substrate proteins include other enzymes, gene transcription factors, scaffolding proteins, receptors, and many other cellular proteins. Thus, kinases can regulate such diverse cellular events as intercellular signaling, cell motility and shape, cell cycle, metabolic pathways, and gene transcription. In addition, kinases themselves may be regulated by intracellular signals, including $Ca^{2+}$ acting via calmodulin. Most cells contain multiple representatives from the diverse family of CaMKs (Figure 2). Generally, CaMKs contain an autoinhibitory domain (AID) that interferes with catalysis by blocking or disrupting the catalytic site. This inhibition is relieved by the binding of $Ca^{2+}$/calmodulin to a domain that overlaps the AID (Figure 2), thus activating the kinase.

CaMKs phosphorylate specific serine or threonine residues in substrate proteins based in part on the surrounding amino acid sequence. Each of the kinases favors target serines or threonines in specific consensus sequences, although their substrate specificities can overlap. For instance, $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) and $Ca^{2+}$/calmodulin-dependent protein kinase IV (CaMKIV) recognize the consensus sequences Hyd-X-Arg-NB-X-Ser/Thr-Hyd and Hyd-X-Arg-X-X-Ser/Thr-X (where Hyd is a hydrophobic residue, NB is a nonbasic residue, and X is any residue), respectively; however, some peptide substrates that fit both of these sequences are phosphorylated by the two kinases with similar affinity. In addition, the catalytic domains of most CaMKs contain acidic residues that are important for interaction with the basic residues in their specific substrates (exceptions are noted below). Based on their substrate specificity, CaMKs can be divided into two general groups: those with narrow substrate specificity and those with broad substrate specificity.

 © 2004, Elsevier Inc. All Rights Reserved. 

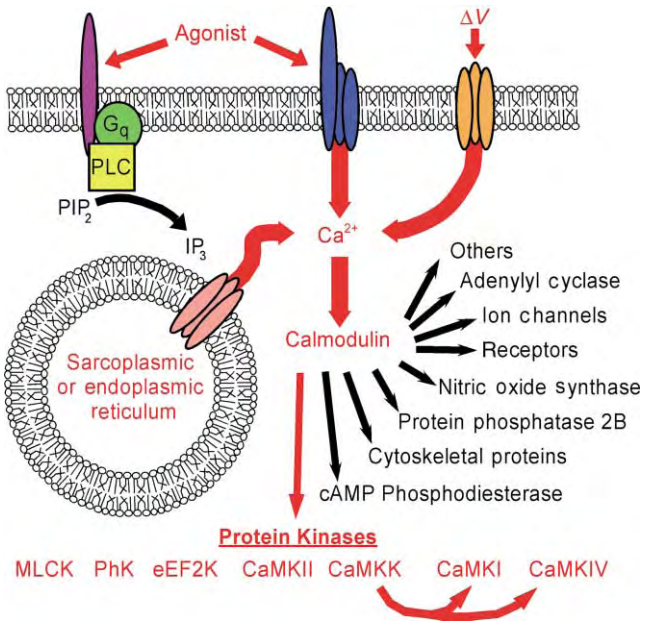



**FIGURE 1** Modulation and effects of intracellular $Ca^{2+}$. $Ca^{2+}$ enters cells via ligand-gated (blue) or voltage-gated (orange) $Ca^{2+}$ channels in the plasma membrane or can be released from sarcoplasmic or endoplasmic reticulum stores via inositol 1,4,5-triphosphate ($IP_3$ (pink)) receptors and/or ryanodine receptors (not shown). $IP_3$ is generated from phosphatidyl-inositol 4,5-bisphosphate ($PIP_2$) by phospholipase C (PLC), which is activated by ligand binding to specific $G_q$ protein coupled receptors (magenta) in the plasma membrane. Cytosolic $Ca^{2+}$ is rapidly extruded from the cell or taken up by the intracellular stores (not shown). The combined actions of these pathways result in spatially localized and transient changes in intracellular $Ca^{2+}$. The ubiquitous $Ca^{2+}$-binding protein calmodulin senses the changes in $Ca^{2+}$ and mediates the activation of multiple intracellular enzymes and other processes, including the family of CaMKs.

# Narrow-Specificity $Ca^{2+}$/Calmodulin-Dependent Protein Kinases

## Myosin Light Chain Kinase

Myosin is the only known *in vivo* substrate of myosin light chain kinase (MLCK), which is found in all vertebrate muscle cells and many nonmuscle tissues. Distinct genes encode two MLCK isoforms. Skeletal muscle MLCK (SkMLCK) is found only in skeletal muscle and contains a catalytic core, an AID, a calmodulin-binding domain, and a short N-terminal extension. The smooth muscle MLCK (SmMLCK), which is expressed in multiple tissues, also contains an actin-binding domain, a fibronectin domain, a PEVK repeat, and a number of immunoglobulin domains that can be varied by alternative splicing. MLCK is normally activated by $Ca^{2+}$ release from the sarcoplasmic reticulum (SR) or endoplasmic reticulum and phosphorylates the regulatory light chain of the molecular motor myosin II, which is sufficient to initiate contraction in smooth muscle or to potentiate the force and speed of contraction in skeletal muscle. Although both forms of MLCK are tightly regulated by calmodulin binding, SmMLCK can be further regulated by phosphorylation. Many kinases, including CaMKII, cyclic AMP-dependent protein kinase (PKA), cyclic GMP-dependent protein kinase, p21-activated kinase, and protein kinase C, can phosphorylate SmMLCK *in vitro* at two serine residues in the calmodulin-binding domain and thus decrease the affinity for calmodulin. In addition,

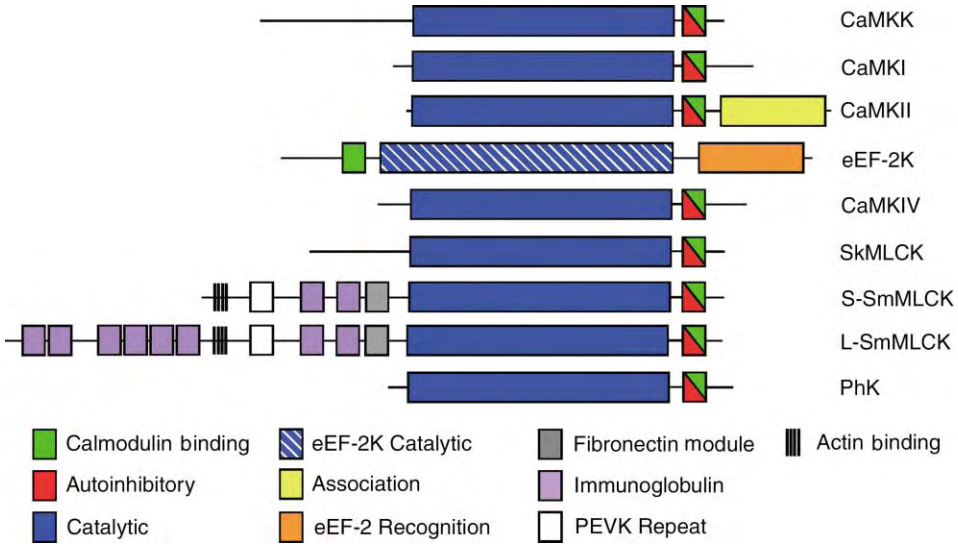



**FIGURE 2** Schematic representation of CaMK domain structures. The key depicts various domains. The kinase domain of eEF-2K, formerly known as CaMKIII, bears no homology to those of the other CaMKs and thus is represented with white hatching. Most family members have multiple isoforms (not shown). Modified from Soderling, T. R., and Stull, J. T. (2001). Structure and regulation of calcium/calmodulin-dependent protein kinases. *Chem. Rev.* **101**, 2341–2351.

two sites outside the catalytic and regulatory regions of SmMLCK can be phosphorylated by members of the mitogen-activated protein kinase family, resulting in an increase in $V_{max}$ but no change in $K_{CaM}$. SmMLCK, but not SkMLCK, is targeted to actin/myosin filaments through interaction with actin subunits.

### Phosphorylase Kinase

Phosphorylase kinase (PhK) phosphorylates and activates glycogen phosphorylase, thus accelerating glycogen degradation. Splice variations and gene duplications result in multiple isoforms, many of which are tissue-specific. Isoforms of PhK are found in muscle, brain, heart, liver, intestines, and many other cell types. The highly abundant liver and muscle enzymes contribute to blood-glucose homeostasis and provide an energy source to facilitate muscle contraction, respectively. Four catalytic $\gamma$-subunits form a 1.3 mDa holoenzyme complex with $\alpha$, $\beta$, and $\delta$ regulatory subunits, each of which is present at four copies per holoenzyme. The three regulatory subunits inhibit the phosphotransferase activity of the catalytic subunit. The $\delta$-subunit is in fact calmodulin, which, unusually, is stably associated with the holoenzyme even at low $Ca^{2+}$ concentrations. $Ca^{2+}$ binding to $\delta$ partially activates PhK and phosphorylation of the $\alpha$- and $\beta$-subunits by PKA potentiates activation. Binding of additional $Ca^{2+}$/calmodulin molecules, or $Ca^{2+}$-bound troponin C, to the holoenzyme maximally activates PhK. PhK associates with a protein termed PTG (protein targeted to glycogen) in muscle, which may facilitate phosphorylation of glycogen phosphorylase *b*, which is also associated with glycogen. PhK also interacts with other proteins in a tissue-specific manner, such as tubulin in rat brain lysates, and is found in membrane preparations from various tissue types.

### Eukaryotic Elongation Factor 2 Kinase

The ubiquitous 90 kDa eukaryotic elongation factor 2 kinase (eEF-2K), also known as CaMKIII, phosphorylates Thr-56 in eEF-2, a protein elongation factor that promotes ribosomal translocation along mRNA during translation. Since eEF-2 is inactivated by this phosphorylation, eEF-2K was initially proposed as a link to reduced global protein synthesis following $Ca^{2+}$ elevation. The eEF-2K contains a calmodulin-binding domain and an eEF-2 recognition domain. However, the catalytic domain of eEF-2K bears no homology to conventional kinases and is a member of the $\alpha$-kinase family. Regulation of eEF-2K is both subtle and complex. In the presence of $Ca^{2+}$ and calmodulin, autophosphorylation of eEF-2K occurs at several serine residues and confers a partially $Ca^{2+}$-independent activity. In addition, phosphorylation by a variety of kinases *in vitro* can both up- and down-regulate eEF-2K activity. Phosphorylation by PKA confers $Ca^{2+}$ independence. Thus, stimulation of $\beta$-adrenergic receptors and subsequent cyclic AMP elevation causes eEF-2K activation and inhibition of protein synthesis in cultured mammalian cells. In addition, studies suggest that slight drops in cellular pH (such as those caused by hypoxia and ischemia) cause activation of eEF-2K. In combination, these data suggest that this kinase may not be a simple, direct link between $Ca^{2+}$ and translation, but rather may function to integrate multiple intracellular signals.

## Broad-Specificity $Ca^{2+}$/Calmodulin-Dependent Protein Kinases

### $Ca^{2+}$/Calmodulin-Dependent Protein Kinase I

$Ca^{2+}$/Calmodulin-dependent protein kinase I (CaMKI) is a ubiquitous cytoplasmic enzyme enriched in brain, liver, and intestine. Three isoforms of the kinase, $\alpha$, $\beta$, and $\gamma$, have been reported and are products of separate genes, each of which may be alternately spliced. CaMKI is an approximately 42 kDa monomer and its catalytic domain shows sequence and structural homologies with those of other serine/threonine kinases. A kinase originally termed CaMKV has been shown to be a splice variant of CaMKI. Goldberg *et al*. crystallized CaMKI, demonstrating that the AID binds and distorts the ATP-binding pocket in the basal state. Binding of $Ca^{2+}$/calmodulin presumably disrupts these interactions. This is the only known structure of a CaMK. A threonine residue in the catalytic domain (activation loop) of CaMKI is then phosphorylated by an upstream kinase, $Ca^{2+}$/calmodulin-dependent protein kinase kinase (CaMKK), resulting in a 10- to 20-fold increase in CaMKI catalytic activity without conferring $Ca^{2+}$/calmodulin independence. However, this phosphorylation can occur only when both CaMKK and CaMKI are bound by $Ca^{2+}$/calmodulin, perhaps permitting synergism in kinase activation. CaMKI phosphorylates synapsin I and cAMP Response Element Binding protein (CREB) *in vitro* and expression of truncated forms of CaMKI activates CREB-dependent gene transcription. However, the physiological substrates and roles of CaMKI remain elusive.

### $Ca^{2+}$/Calmodulin-Dependent Protein Kinase II

CaMKII is a ubiquitous kinase that has been implicated in the regulation of diverse physiological processes, such

as gene transcription, neuronal plasticity, exocytosis, and metabolism. Four separate genes encode the $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-isoforms of CaMKII. Each isoform is subjected to alternative mRNA splicing, resulting in over 20 known variants of the kinase, many of which have specific cellular and tissue distributions. In addition to catalytic, AID, and calmodulin-binding domains, most CaMKII isoforms contain a C-terminal association domain that facilitates the formation of a holoenzyme structure. The $\alpha$-isoform forms a double hexameric ring-shaped holoenzyme and other isoforms appear to form similar multimeric holoenzymes, which may contain multiple isoforms or splice variants. The holoenzyme structure of CaMKII facilitates complex regulatory properties. Each subunit is independently activated by $Ca^{2+}$/calmodulin binding. When adjacent subunits in a holoenzyme bind $Ca^{2+}$/calmodulin, there is a trans-autophosphorylation at Thr-286, resulting in $Ca^{2+}$ independence and also a 1000-fold increase in affinity for calmodulin. The kinase can be inactivated only by the actions of cellular protein phosphatases. Additional autophosphorylation at Thr-305 and Thr-306 in the calmodulin-binding domain occurs in the absence of $Ca^{2+}$/calmodulin. Thr-305/Thr-306 phosphorylation interferes with calmodulin binding, blocking $Ca^{2+}$/ calmodulin-dependent activation.

The number of subunits in a holoenzyme that become active and autophosphorylated at Thr-286 is directly related to the $Ca^{2+}$ concentration. Thus, CaMKII is able to "decode" the frequency and amplitude of $Ca^{2+}$ spikes. This unique capacity has been proposed to play an important role in prolonged signaling after transient changes in $Ca^{2+}$, such as may be involved in learning and memory. Consistent with this idea, transgenic mice in which the CaMKII$\alpha$ gene has been mutated such that the kinase cannot autophosphorylate at Thr-286 are deficient in spatial learning tasks.

CaMKII can be found in the cytosol, in the nucleus, and associated with various organelles or membranes, depending on the cell type examined. The identity of CaMKII splice variants expressed is believed to play a role in modulating subcellular localization, although few specific examples have been documented. For example, one variant of CaMKII$\delta$ contains a nuclear localization sequence and has been shown to regulate gene transcription in cardiac myocytes. Variable subcellular localization can also be attributed in part to the large number of proteins with which CaMKII may associate, including several neuronal proteins (e.g., *N*-methyl-D-aspartate (NMDA) receptor, $\alpha$-actinin, synapsin I, and densin-180) and contractile/motor proteins (e.g., actin and myosin V). Binding of CaMKII to these proteins may position the kinase close to $Ca^{2+}$ sources, and/or close to specific substrates, and may modulate its availability to cellular phosphatases, thereby affecting the rate of inactivation. One splice variant of the CaMKII$\alpha$ gene ($\alpha$KAP) functions as a CaMKII anchoring protein. $\alpha$KAP lacks a catalytic domain but forms hetero-multimers with the conventional isoforms through an intact association domain and associates with the SR in muscle via an N-terminal hydrophobic region.

At last count, CaMKII phosphorylates at least 50 distinct proteins *in vitro* and many of these are likely to be physiologically relevant. However, relatively few of these have been formally shown to be phosphorylated in intact cells under physiologically relevant conditions. One of the best characterized substrates is the neuronal L-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-type glutamate receptor; phosphorylation of this receptor at Ser-831 by CaMKII plays an important role in the regulation of synaptic transmission. In neuronal presynaptic terminals, phosphorylation of synapsin 1 by CaMKII modulates synaptic vesicle movement. In the heart, CaMKII plays a critical role in feedback regulation of $Ca^{2+}$ dynamics by regulating $Ca^{2+}$ entry via L-type voltage-gated $Ca^{2+}$ channels, by phosphorylating ryanodine receptors to regulate SR $Ca^{2+}$ release, and by phosphorylating phospholamban at Thr-17, thereby regulating SR $Ca^{2+}$ uptake via the $Ca^{2+}$-ATPase.

## $Ca^{2+}$/Calmodulin-Dependent Protein Kinase IV

$\alpha$CaMKIV is a monomeric kinase expressed largely in neuronal tissues, testes, and T cells, whereas the $\beta$CaMKIV splice variant is differentially expressed in the cerebellum during development. Like CaMKI, CaMKIV is initially activated by calmodulin binding and is further activated when phosphorylated by CaMKK in its activation loop. However, combined with a subsequent N-terminal autophosphorylation, the activation by CaMKK eventually leads to $Ca^{2+}$-independent activity of CaMKIV. An additional autophosphorylation at Ser-332 of the calmodulin-binding domain prevents further $Ca^{2+}$/calmodulin binding in a manner analogous to CaMKII autophosphorylation at Thr-305 and Thr-306. CaMKIV contains a nuclear localization sequence and it is thought to be responsible for $Ca^{2+}$-dependent phosphorylation of a variety of transcription factors (e.g., CREB, serum response factor, and myocyte enhancer factor 2 (MEF2)) and regulatory factors, such as the CREB-binding protein. In the cytosol, CaMKIV phosphorylates oncoprotein 18, preventing its association with tubulin. Protein phosphatase 2A (PP2A), a ubiquitous multimeric protein phosphatase, associates with and dephosphorylates CaMKIV, forming a

self-regulating complex that may be a prototype for phosphatase–kinase cross-talk in cell signaling.

### $Ca^{2+}$/Calmodulin-Dependent Protein Kinase Kinase

CaMKK, originally characterized as an "activating factor" from brain extracts, can dramatically increase the activity of CaMKI and CaMKIV when incubated in the presence of $Ca^{2+}$/calmodulin, $Mg^{2+}$, and ATP. Two isoforms ($\alpha$ and $\beta$) of CaMKK have been cloned and their domain organization and function are similar to those of other $Ca^{2+}$/calmodulin-dependent kinases. However, CaMKK is unique in its method of substrate recognition, lacking acidic residues to recognize basic residues near its preferred phosphorylation sites. Instead, this kinase contains an arginine- and proline-rich (RP) insert that is important for phosphorylation of CaMKI and CaMKIV and $Ca^{2+}$/calmodulin must be bound to both CaMKK and the substrate (CaMKI or CaMKIV) for phosphorylation to occur. CaMKK also phosphorylates protein kinase B both *in vitro* and in cultured cells, although this does not require the RP domain. Regulation of CaMKK also occurs via other kinases, such as PKA, which phosphorylates CaMKK in its calmodulin-binding domain, resulting in the inhibition of $Ca^{2+}$/calmodulin-induced activity.

## Summary

Repetitive transient changes in $Ca^{2+}$ concentration in every mammalian cell are sensed by a number of different $Ca^{2+}$/calmodulin-dependent protein kinases. Although many of these kinases share a great deal of functional homology, there are diverse regulatory nuances. Moreover, some of these kinases phosphorylate a number of different substrates, whereas others have only one principal substrate. The specific response of individual cells to changes in intracellular $Ca^{2+}$ concentration likely depends on the subset and specific isoforms of $Ca^{2+}$/calmodulin-dependent kinases expressed in that cell. For example, in hippocampal neurons, synaptic activity increases $Ca^{2+}$ levels in dendritic regions. In the short term, dendritic $Ca^{2+}$ locally activates CaMKII, which phosphorylates pre-existing synaptic glutamate receptors. However, over a longer time frame, $Ca^{2+}$ signals the activation of nuclear CaMKIV and thus the transcription of specific genes. It is the

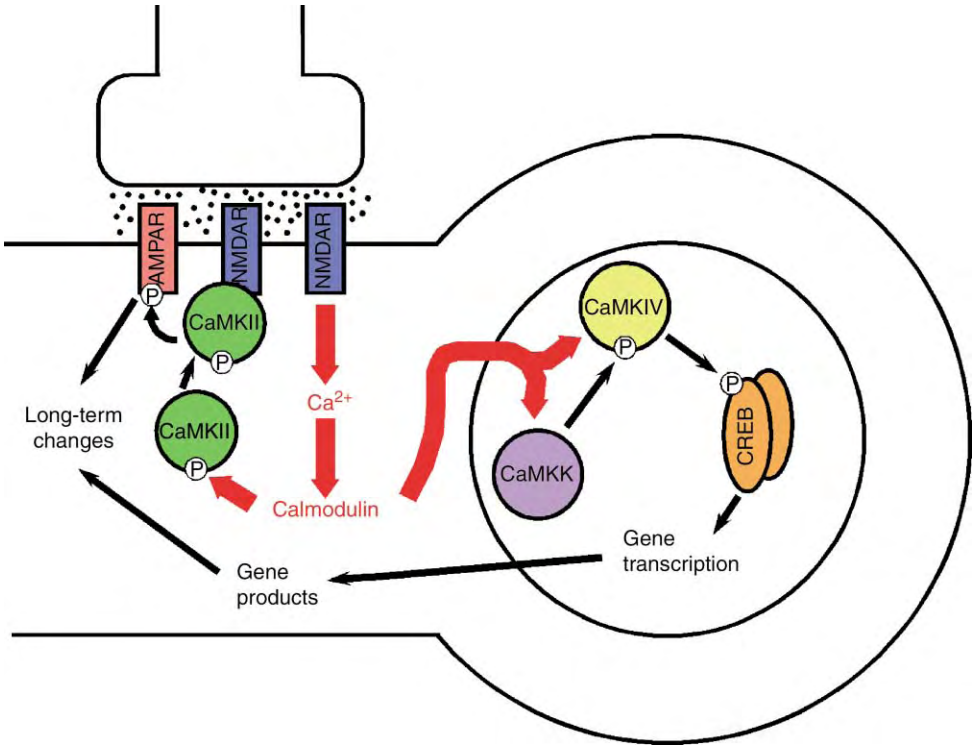



**FIGURE 3** Modulation of neuronal function by activation of CaMKII and CaMKIV. Glutamate release from presynaptic terminals activates NMDA- (blue) and AMPA- (red) type glutamate receptors on the postsynaptic neuron. The NMDA receptor (NMDAR) is a ligand-gated $Ca^{2+}$ channel. The elevated local intracellular $Ca^{2+}$ in the dendrites activates CaMKII, which phosphorylates AMPA-type glutamate receptors, potentiating synaptic transmission. $Ca^{2+}$ elevation via NMDA- and voltage-gated $Ca^{2+}$ channels (not shown) also signals to the nucleus to activate CaMKK and CaMKIV, driving the transcription of specific genes. The dual CaMKII and CaMKIV signals combine to promote long-term changes in synaptic function. AMPAR, AMPA receptor.

combined actions of CaMKII and CaMKIV that result in long-lasting changes in the synaptic response of these neurons that may underlie learning and memory (Figure 3). However, much work remains to be performed in identifying substrates and physiological roles of the various $Ca^{2+}$/calmodulin-dependent protein kinases in other cells and tissues.

## See Also the Following Articles

Calcium/Calmodulin-Dependent Protein Kinase II • Calcium Signaling: Cell Cycle • Mitochondrial Membranes, Structural Organization • Protein Kinase C Family

## Glossary

**autoinhibitory domain (AID)** The region of a $Ca^{2+}$/calmodulin-dependent protein kinase that binds and inhibits the catalytic domain. Inhibition is relieved by interaction with calmodulin and in some cases is prevented by subsequent autophosphorylation.

**calmodulin** A small cytosolic protein that binds four $Ca^{2+}$ ions and subsequently associates with and modulates many important proteins, including some protein kinases.

**catalytic domain** The region of an enzyme responsible for activity; in protein kinases, this domain transfers the $\gamma$-phosphate from ATP to the target residue.

**protein kinase** An enzyme that transfers the terminal ($\gamma$) phosphate from ATP to a serine, threonine, or tyrosine residue of a substrate protein.

**protein phosphatase** An enzyme that hydrolyzes a phosphate group from a serine, threonine, or tyrosine residue of a phosphoprotein substrate.

## Further Reading

Berridge, M. J., Lipp, P., and Bootman, M. D. (2000). The versatility and universality of calcium signaling. *Nature Rev.* **1**, 11–20.

Braun, A. P., and Schulman, H. (1995). The multifunctional calcium/calmodulin-dependent protein kinase: From form to function. *Annu. Rev. Phyiol.* **57**, 417–445.

Brushia, R. J., and Walsh, D. A. (1999). Phosphorylase kinase: The complexity of its regulation is reflected in the complexity of its structure. *Front. Biosci.* **4**, 618–641.

Goldberg, J., Nairn, A. C., and Kuriyan, J. (1996). Structural basis for the autoinhibition of calcium/calmodulin-dependent protein kinase I. *Cell* **84**, 875–887.

Hook, S. S., and Means, A. (2001). $Ca^{2+}$/CaM-dependent kinases: From activation to function. *Annu. Rev. Pharmacol. Toxicol.* **41**, 471–505.

Kamm, K. E., and Stull, J. T. (2001). Dedicated myosin light chain kinases with diverse cellular functions. *J. Biol. Chem.* **276**, 4527–4530.

Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nature Rev. Neurosci.* **3**, 175–190.

Pavur, K. S., Petrov, A. N., and Ryanazov, A. G. (2000). Mapping the functional domains of elongation factor-2 kinase. *Biochemistry* **39**, 12216–12224.

Proud, C. G., and Denton, R. M. (1997). Molecular mechanisms for the control of translation by insulin. *Biochem. J.* **328**, 329–341.

Soderling, T. R., and Stull, J. T. (2001). Structure and regulation of calcium/calmodulin-dependent protein kinases. *Chem. Rev.* **101**, 2341–2351.

## Biography

A. J. Robison is a graduate student in the Molecular Physiology and Biophysics Department at Vanderbilt University. He received a B.S. in biology with honors from Rhodes College in 1999. He is studying the subcellular anchoring of neuronal CaMKII in the laboratory of Dr. Roger Colbran.

Roger. J. Colbran is an Associate Professor of Molecular Physiology and Biophysics, a member of the Center for Molecular Neuroscience, and an Investigator at the John F. Kennedy Center for Research on Human Development at Vanderbilt University. He earned a Ph.D. from the University of Newcastle upon Tyne (United Kingdom) and received postdoctoral training at Vanderbilt University. His principal research interest is in understanding calcium-dependent signaling events in neurons, especially as this relates to the regulation of synaptic transmission, and in cardiomyocytes.

# Calcium-Binding Proteins: Cytosolic (Annexins, Gelsolins, $C_2$-Domain Proteins)

**Joachim Krebs**
*Swiss Federal Institute of Technology, Zurich, Switzerland*

**Calcium is one of the most common elements on Earth, and it is the fifth most abundant element of the human body. Inside cells, calcium is a very versatile second messenger involved in the regulation of a variety of different cellular processes. This pivotal role of calcium is made possible due to the binding of $Ca^{2+}$ to a great variety of different calcium-binding proteins. Next to the well-studied family of the so-called EF-hand containing proteins, a number of other calcium-binding proteins became known in recent years. These proteins are composed of a number of repeat units containing a variety of different $Ca^{2+}$-binding sites. The families of annexins, gelsolins, and $C_2$-domain containing proteins described in this article bind to different membranous or cytoskeletal structures in a $Ca^{2+}$-dependent manner, and are involved in a number of different cellular functions.**

## Annexins

The family of intracellular $Ca^{2+}$-binding proteins called annexins is soluble, amphipathic proteins that bind to membranes containing negatively charged phospholipids in a $Ca^{2+}$-dependent manner. They are called annexins since they bring or hold together different cellular structures, in particular membranes. The close to 200 different annexin proteins known to date are widespread in the animal and plant kingdoms, and have been claimed to be involved in a variety of cellular functions such as interaction with the cytoskeleton, membrane fusion, anticoagulation, signal transduction, or phospholipase inhibition. The primary structure of the annexins contains four or eight (annexin VI) conserved repeat units of ~75 amino acids in length, the protein core, which are separated by intervening sequences of variable length. These repeat units probably originated from gene duplications, a view which is corroborated by the evolutionary conservation of the intron–exon boundary positions. Most annexins containing four repeat units are comprised of 12–15 exons resulting in a quite variable amino-terminal part of the different annexins. Most alternative splicing sites have been located within exons encoding the variable amino-terminal parts of annexins. Since these parts are also the locations containing motifs for binding partners or for post-translational modifications, thus alternative splicing may contribute to the regulation of annexin function.

After the first determination of an annexin crystal structure (annexin V) by Huber and his co-workers, a number of additional annexin structures from different sources (including those from lower eukaryotes and plants) have been reported. From these structures it can be concluded that the conserved repeat units are packed into an $\alpha$-helical disk, as first described for annexin V, which is almost entirely $\alpha$-helical. It consists of five $\alpha$-helices bundled into a right-handed super-helix. On the basis of this structure it was proposed that annexin V functions as a calcium channel, and some experiments using an *in vitro* reconstituted system seem to support a voltage-gated mechanism. This property is connected with the structure of the annexin core, which is shared by most annexins. Meanwhile, for most annexins a $Ca^{2+}$ channel activity has been demonstrated in artificial bilayer systems, but never *in vivo*; therefore, the physiological relevance of these observations is questionable.

In contrast to the EF-hand containing $Ca^{2+}$-binding proteins, the ligands coordinating calcium in the annexins are not adjacent in sequence. Several calcium-binding sites seem to exist in annexins, two invariably in repeats II and IV, one in either repeat I or III, but annexins may contain as many as 10–12 $Ca^{2+}$-binding sites along the membrane-binding surface of the protein. The sites with the highest $Ca^{2+}$ affinity are structurally related to the $Ca^{2+}$- and phospholipid-binding site of phospholipase $A_2$. The calcium ion is heptacoordinated with ligands organized in a pentagonal bipyramidal arrangement with ligating oxygens provided mainly from peptide carbonyls and water molecules, together with a single side-chain oxygen from a distant part of the sequence. Replacement of this latter

 © 2004, Elsevier Inc. All Rights Reserved. 

"capping" residue, which is usually acidic, with alanine precludes $Ca^{2+}$ binding at the site. Once bound to membranes, many annexins oligomerize to form highly ordered two-dimensional arrays that have been shown to strongly influence *in vitro* membrane properties, e.g., increased rigidity.

An interesting feature is the role of Trp185 in the $Ca^{2+}$-binding site of repeat III of annexin V (see Figure 1). As suggested by Seaton and others, a $Ca^{2+}$-dependent exposure of Trp185, which is buried within the protein core in the absence of calcium, may facilitate the interaction between annexin V and the phospholipid bilayer. This view has been supported by fluorescence data and by mutagenesis experiments in which replacement of Trp185 by alanine decreased the annexin V membrane-binding affinity.

Annexins have long been known as targets for post-translational modifications. In fact, annexin II was originally isolated as a major substrate of the *src*-encoded protein kinase, and annexin I was long known to be phosphorylated by the tyrosine kinase activity of the epidermal growth factor (EGF) receptor, suggesting that annexins I and II could be used as coupling factors between growth factor receptors and their cellular targets, since these phosphorylations alter the $Ca^{2+}$-dependent binding of annexin I and II to membranes. In addition to the phosphorylation by different tyrosine kinases, also serine/threonine kinases have been identified to phosphorylate annexins. As a result, annexin phosphorylation often leads to an altered susceptibility towards proteolysis. On the other hand, phosphorylation of annexin II by different kinases interferes with its ability to form stable heterotetrameric complexes with p11, a member of the S100 family (S100A10), since these phosphorylation sites are located – like in the other annexins – within the variable amino-terminal domain, which comprises also binding motifs to interact with other proteins. Due to the binding of p11 (S100A10) the $Ca^{2+}$-sensitivity of the complex is increased by 3 orders of magnitude, and enables the heterotetrameric complex to bind simultaneously to two different membrane surfaces through the two annexin II cores. Next to the interaction between annexin II and S100A10 (p11) two other complexes between an annexin and an EF-hand containing protein of the S100 family may exist, i.e., interactions between annexin XI and S100A6 and annexin I and S100A11, respectively, but in contrast to annexin II/S100A10 the existence of the latter complexes *in vivo* has not been described to date.

In spite of extended studies in many laboratories over the years, the precise function of individual annexins is not yet understood. The accumulated data suggest that due to the many ways that annexins interact with different membranes in a $Ca^{2+}$-dependent manner, annexins may participate in $Ca^{2+}$ signaling as effectors, mediators or even as regulators, but to clarify their biological activities unambiguously, this has to await further experiments. However, *in vivo* experiments from knockout and transgenic mice are beginning to shed some light on annexin function.

## Gelsolin

Gelsolin belongs to the superfamily of actin-binding proteins (ABPs) expressed in all eukaryotes. It is a multifunctional protein-binding actin in a $Ca^{2+}$-dependent

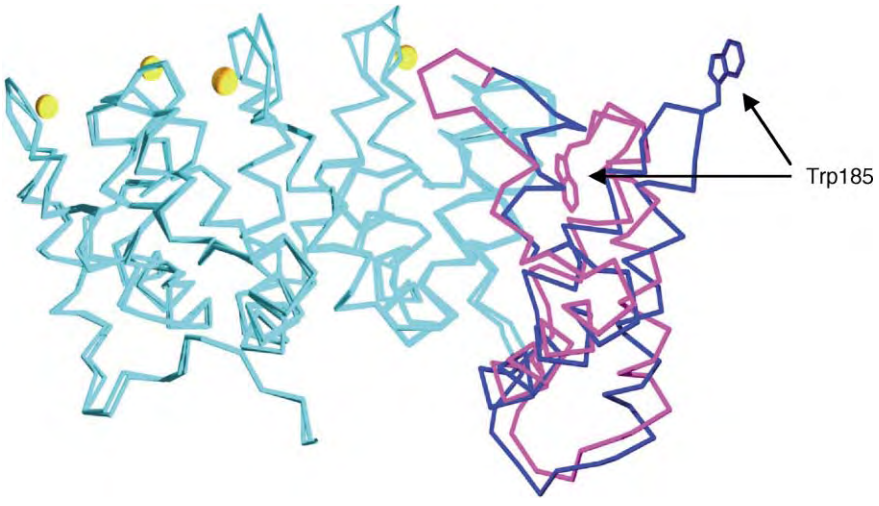



**FIGURE 1** Superposition of $\alpha$-carbon backbones of annexin V in the $Ca^{2+}$-free and the $Ca^{2+}$-bound form. Domains 1, 2, and 4 are light green, both in the $Ca^{2+}$-free and in the $Ca^{2+}$-bound form. Domain 3 is shown in magenta ($Ca^{2+}$-free, trp185 buried) and in blue ($Ca^{2+}$-bound, trp185 exposed). (Courtesy of Barbara Seaton.)

manner, and is composed of up to six repeats of 120–150 amino acids. To the gelsolin family belong proteins such as villin, adseverin, CapG, flil, and others. Villin, for example, is a six gelsolin-domain ABP regulating actin assembly in microvilli, whereas CapG is a smaller, three-domain gelsolin analogue that responds to $Ca^{2+}$ and phosphatidylinositol-(4,5)-diphosphate under conditions where gelsolin is ineffective.

Alternative transcription initiation and selective RNA processing produces two isoforms of gelsolin from the same gene. One isoform of 80 kDa is located intracellular, whereas the other extracellular, slightly larger (83 kDa) protein is derived by alternative splicing, which results in a short amino-terminal elongation of the protein. The intracellular gelsolin is involved in cell motility regulating actin function, whereas extracellular gelsolin can act as an actin-scavenging system to prevent the polymerization of actin released after cell death.

Gelsolin consists of six repeat units (G1–6). The units are organized in two clusters of similar architecture, and are connected by a flexible linker of ~50 amino acids that may be cleaved by caspase-3, thereby cleaving the actin-binding domain of gelsolin from its calcium-binding domain. Caspase-3 cleavage and separation of subdomains of gelsolin coincides with plasma membrane blebbing, one of the characteristics of apoptosis. Apoptosis could also be induced by the overexpression of the amino-terminal half of gelsolin, whereas neutrophils of gelsolin-null mice have a delayed onset of apoptosis.

The structure of gelsolin in the absence of $Ca^{2+}$ has been determined at 2.5Å resolution by Robinson and his co-workers (Figure 2). The amino-terminal half can bind to two actin monomers independent of calcium, whereas the carboxy-terminal half binds a single actin in a $Ca^{2+}$-dependent manner.

A detailed mechanism for the regulation of gelsolin activity by $Ca^{2+}$ has recently been proposed. This was made possible by comparing the $Ca^{2+}$-free structure of nonactive gelsolin (Figure 2) with the recently solved structure of the active form of $Ca^{2+}$-bound gelsolin complexed to actin (see Figure 3). In this latter structure consisting of the C-terminal half of gelsolin, i.e., G4–6, bound to monomeric actin two classes of $Ca^{2+}$-binding sites have been identified: type-1 sites bind calcium in a coordination sphere shared by actin and gelsolin, whereas type-2 sites are identified only in gelsolin, i.e., located within G5 and G6. Due to conservation of the ligating residues within all gelsolin repeats, type-2 $Ca^{2+}$-binding sites should exist in all six domains of gelsolin. In total, gelsolin should be able to bind eight calcium ions in the active gelsolin–actin complex, i.e., two in type-1 sites and six in type 2.

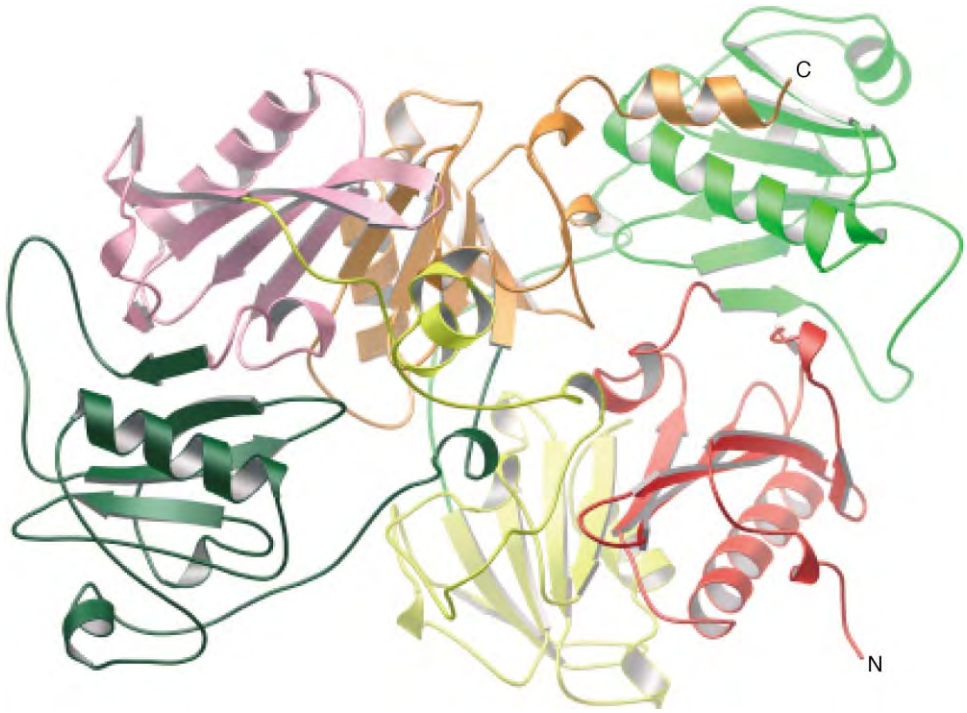



**FIGURE 2** A ribbon diagram of the structure of gelsolin in the absence of calcium. The following color code for the different segments (G1–6) has been used: G1 (containing the amino terminus) red; G2, light green; G3, yellow; G4, magenta; G5, dark green; G6, (containing the carboxy terminus) gold. (Reproduced from Burtnick, L. D., Koepf, E. K., Grimes, J., Jones, E. Y., Stuart, D. I., McLaughlin, P. J., and Robinson, R. C. (1997). The crystal structure of plasma gelsolin: Implications for actin severing, capping and nucleation. *Cell* **90**, 661–670, with permission from Elsevier.)

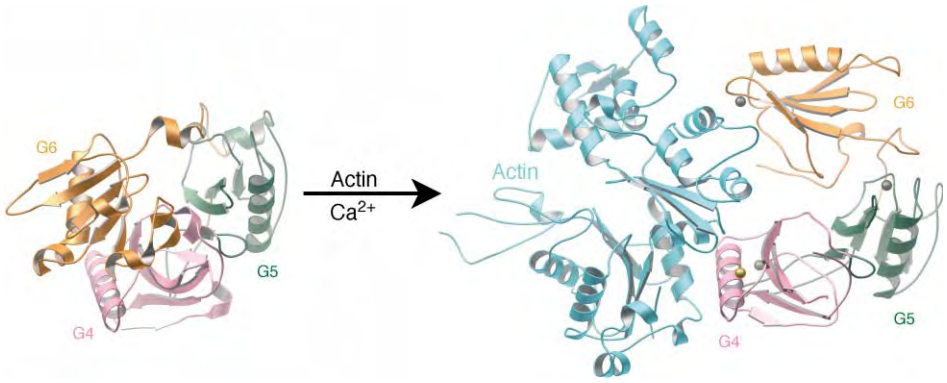



**FIGURE 3** Ribbon diagram of gelsolin domains G4–G6 demonstrating the influence of binding $Ca^{2+}$ and actin (shown in cyan) on the conformational changes of the protein. The same color code as in Figure 2 is used for domains G4–G6. The left-hand panel shows G4–G6 in a $Ca^{2+}$-free conformation (see Figure 2), whereas the right hand panel depicts the actin and $Ca^{2+}$-bound form of G4–6. (Reproduced from Choe, H., Burtnick, L. D., Mejillano, M., Yin, H. L., Robinson, R. C., and Choe, S. (2002). The calcium activation of gelsolin: Insights from the 3Å structure of the G4–G6/actin complex. *J. Mol. Biol.* **324**, 691–702, with permission from Elsevier.)

The latter are proposed to facilitate structural rearrangements within gelsolin as part of the activation and actin-binding process.

In the absence of $Ca^{2+}$ the six repeat units of gelsolin provide a very compact globular structure, thus blocking the actin-binding helices of the appropriate sub-domains. Therefore, in a first step, this compact globular arrangement of the six sub-domains has to be opened up. As suggested by H. Choe and co-workers in 2002, binding of $Ca^{2+}$ first to G6 should induce a conformational rearrangement in which G6 is flipped over G5 to tear apart the continuous $\beta$-sheet core of G4 and G6, thereby unmasking the actin-binding site on G4, and hence permit binding to actin strands (see Figure 3). Similar events should also occur in the N-terminal half of gelsolin, i.e., $Ca^{2+}$-binding to G3, flipping over G2 to open up the actin-binding site of G1. Finally, to tighten up the gelsolin–actin complex, $Ca^{2+}$ binds to the two type-1 sites shared between actin and gelsolin, which might explain the very high affinity of gelsolin to actin ($K_d$ 50 nM).

Gelsolin-null mice show normal embryonic development, but suffer subtle changes. This suggests the need of gelsolin for cell motility during processes such as hemostasis leading to reduced platelet function, inflammation leading to delayed neutrophil migration, or during wound healing, which would lead to reduced movement of fibroblasts. In this context it is interesting to note that it was demonstrated that during development of zebrafish embryos gelsolin is required for dorsoventral patterning. Inhibition of gelsolin expression that starts to be expressed already at the two-cell stage resulted in ventralized phenotypes, some of which lacked brain or eyes. These phenotypes could be rescued by injecting zebrafish gelsolin mRNA or even by injecting human gelsolin protein. These data indicate that gelsolin may have at least two separate functions: a structural role for the cytoskeletal/cell motility apparatus, and a regulatory role during development.

## $C_2$-Domain Proteins

Interaction of proteins, intracellularly or extracellularly, often occurs via different binding modules or domains such as SH2, SH3, WW, PDZ, or $C_2$-domains. These modules are formed by folding domains consisting of 100–150 residues with different binding properties, that is, SH2-domains interact with phosphotyrosine-containing sequences, SH3- and WW-domains with proline-rich sequences, PDZ-domains with C-terminal sequences to link multiprotein complexes to the cytoskeleton, and $C_2$-domains with phospholipids, some in a $Ca^{2+}$-dependent, some in a $Ca^{2+}$-independent, manner. $C_2$-domains consist of about 120–130 amino acid residues. They were first identified in protein kinase C. More than 100 $C_2$-domain containing proteins can now be identified in current data banks.

Most proteins containing $C_2$-domains are linked either to signal transduction pathways or are involved in membrane traffic. The former proteins either generate lipid second messengers (e.g., phospholipase A2, phospholipase C, or phosphatidylinositol-3-kinase), phosphorylate proteins (e.g., protein kinase C), or ligate ubiquitin (e.g., Nedd4). Those proteins involved in membrane traffic include, for instance, the Rab-binding proteins rabphilin and RIM, which are involved in regulating the exocytosis of secretory vesicles. However, the best-characterized protein of

this category is synaptotagmin, which will be discussed in more detail.

Synaptotagmin I is a transmembrane protein belonging to a family of more than ten members which contain two $C_2$-domains, $C_2A$ and $C_2B$, comprising most of the cytoplasmic region of synaptotagmins. The $C_2$-domains contain $Ca^{2+}$-binding domains of non-EF-hand character. Synaptotagmin I is found in synaptic vesicles, and is believed to act as the major $Ca^{2+}$-sensor of exocytosis and neurotransmitter release. Important in this respect is the $Ca^{2+}$-dependent binding of synaptotagmin to syntaxin, a member of the SNARE family of membrane proteins, involved in vesicle transport mechanisms.

$C_2$-domains have been suggested to be responsible for binding to membranes in response to $Ca^{2+}$. Sudhof and his co-workers determined the first structure of a $C_2$-domain, the $C_2A$-domain of synaptotagmin I (see Figure 4). The $C_2$-domain consists of a $\beta$-sandwich of two four-stranded $\beta$-sheets (Figure 4A). The eight $\beta$-strands are connected by three loops at the top that bind $Ca^{2+}$ in the form of a cluster, primarily through oxygen of aspartate side chains serving as bidentate ligands, and by four loops at the bottom lacking

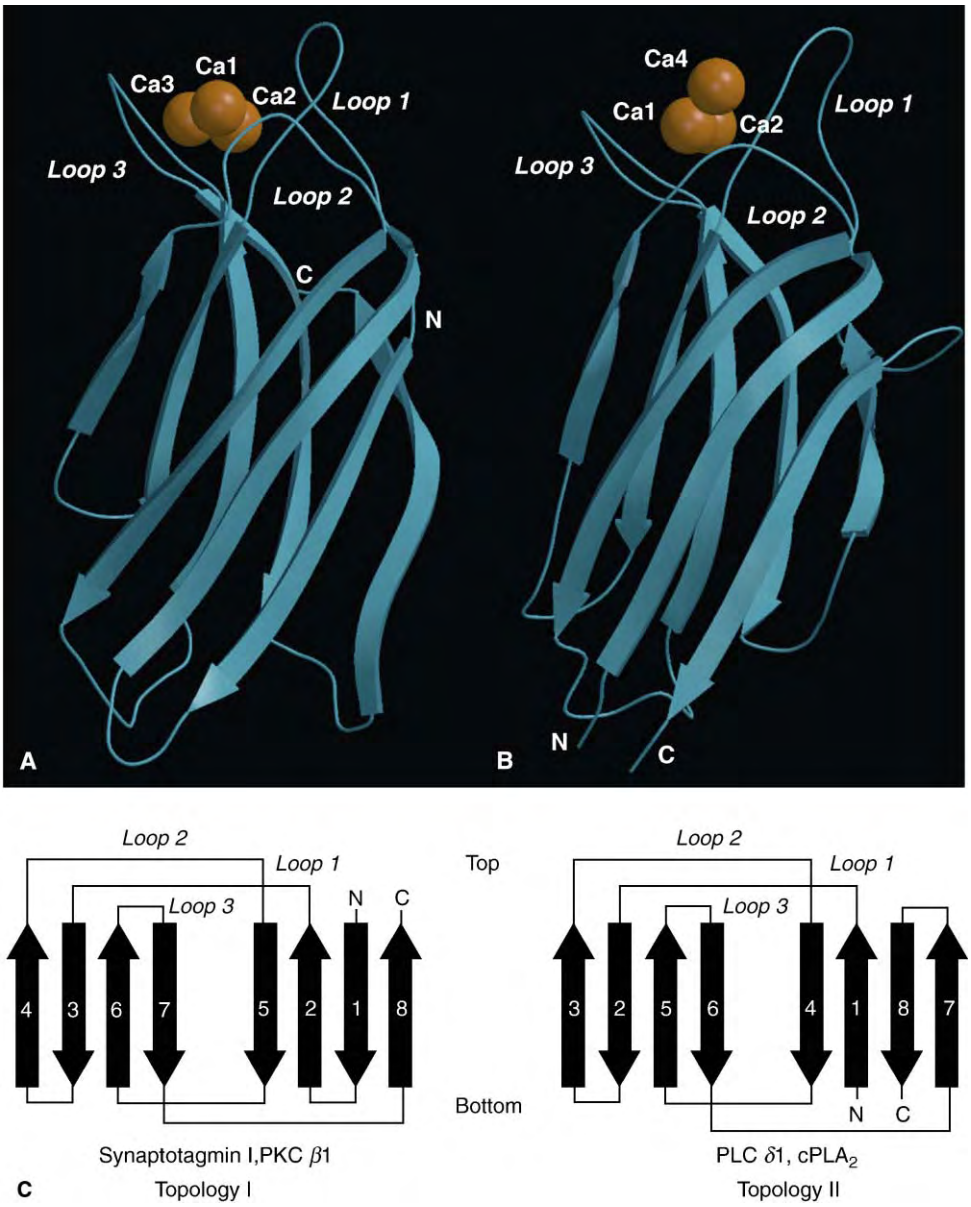



**FIGURE 4** Ribbon diagrams of the structures of the $C_2A$-domain of synaptotagmin I (A) and of the $C_2$-domain of phospholipase C$\delta$1(B). (C) A schematic drawing of the $\beta$-strand topologies of the two structures. The locations of the $Ca^{2+}$ clusters (orange) are indicated. (Reproduced from Rizo, J., and Südhof, T. C. (1998). $C_2$-domains, structure and function of a universal $Ca^{2+}$-binding domain. *J. Biol. Chem.* **273**, 15879–15882, with permission of the American Society for Biochemistry & Molecular Biology.)

$Ca^{2+}$-binding sites. In contrast to $Ca^{2+}$-binding to EF-hand type of sites, which causes substantial conformational changes, $Ca^{2+}$-binding to sites of $C_2$-domains leads to structural stabilization rather than backbone rearrangements.

Structural determination of other $C_2$-domains revealed similar designs, but significant differences in the topology of the arrangement of the $\beta$-strands (see Figure 4C). It could be shown that the structure of the $C_2$-domain of protein kinase C$\beta$ is very similar to synaptotagmin I (topology I), whereas the topological arrangement of the structure of the $C_2$-domain of phospholipase C$\delta$ is significantly different (topology II) (see Figure 4C). Phospholipase C$\delta$ contains several protein modules including EF-hand $Ca^{2+}$-binding domains, which provides evidence for the existence of proteins containing EF-hand and non-EF-hand $Ca^{2+}$-binding sites.

An interesting finding concerning important functional differences between $Ca^{2+}$-binding sites of $C_2$A- and $C_2$B-domains of synaptotagmin has recently been reported. It has been shown that by mutating an aspartate essential for $Ca^{2+}$-binding to the $C_2$A-domain into an asparagine, this domain lost its $Ca^{2+}$-dependent binding to phospholipids or to syntaxin. However, introducing such a mutated synaptotagmin into the germline of *Drosophila* lacking synaptotagmin I that were severely impaired in neurotransmitter release, the mutated synaptotagmin I could fully rescue this defect, indicating that $Ca^{2+}$-binding to the $C_2$A-domain of synaptotagmin is not essential for neurotransmitter release. However, by replacing an aspartate for asparagine essential for $Ca^{2+}$-binding in the $C_2$B-domain, this mutated protein could not rescue such a defect in a similar set of experiments suggesting a significant functional difference in $Ca^{2+}$-binding between the $C_2$A- and $C_2$B-domains of synaptotagmin I.

In another $C_2$-domain containing protein, Nedd4, it was demonstrated that binding of $Ca^{2+}$ to the $C_2$-domain was responsible not only for the localization of the protein but also for part of its function. Nedd4, the neuronal precursor cell-expressed developmentally down-regulated four protein, is a multimodular ubiquitin protein ligase. This enzyme is involved in controlling the turnover of membrane proteins. It was shown that localization of Nedd4 to the apical region of polarized epithelial cells was dependent on the $Ca^{2+}$-binding to its $C_2$-domain. Here an important target of Nedd4 is the $Na^+$-channel (ENaC), which plays a critical role in $Na^+$ homeostasis of epithelial cells. However, the $C_2$-domain of Nedd4 was not required to inhibit EnaC. On the other hand, it was shown that Nedd4 lacking its $C_2$-domain was still able to ligate ubiquitin to appropriate membrane protein targets such as Gap1, the general amino acid permease, but subsequent internalization by endocytosis, a prerequisite for the downregulation of membrane receptor proteins, was impaired.

## See Also the Following Articles

Actin-Capping and -Severing Proteins • Calcium-Modulated Proteins (EF-Hand) • Phospholipase $A_2$ • Phospholipase C • Ribozyme Structural Elements: Group I Introns

## Glossary

**actin** A eukaryotic protein that has the capability to form thin helical filaments.

**alternative splicing** Alternate usage of particular exons to create isoforms of a given protein.

**amphipathic proteins** Proteins composed of helices containing both hydrophobic and hydrophilic amino acid residues.

**apoptosis** Apoptosis (or programmed cell death) is derived from a Greek word describing the shedding of leaves from trees. During apoptosis, the cell responds to specific physiological or developmental signals undergoing a regulated, well-programmed series of events which will lead to its death and its removal from the organism.

**cytoskeleton** Complement of actin filaments, microtubules, and intermediate filaments forming a network in the cytoplasm.

**EF-hand proteins** Term coined by R.H. Kretsinger to describe the helix–loop–helix calcium-binding domains of specific proteins. The highly conserved motif (first described on the basis of the crystal structure of parvalbumin containing six helices, A–F) in which certain amino acids are invariant consists of two helices enclosing the $Ca^{2+}$-binding loop. As a model, the forefinger and the thumb of the right hand can resemble the two helices (e.g., E and F of the second $Ca^{2+}$-binding domain of parvalbumin) and the bent midfinger the enclosed loop, hence the EF-hand.

**exon** Segments of a eukaryotic gene preserved in the mature messenger RNA.

**intron** Segments of a gene transcribed into the precursor RNA but excised by RNA splicing before the mature RNA is exported from the nucleus into the cytoplasm.

**post-translational modification** Enzymatic modification such as acetylation, phosphorylation, myristoylation, or ubiquitination of proteins regulate their activity, topology, or degradation.

**second messenger** Intracellular small molecules (e.g., cyclic nucleotides or inositol polyphosphates) or ions such as $Ca^{2+}$ or gases such as NO, indispensable for the transduction of signals converting extracellular stimuli – e.g., raised by hormones (= primary messengers) – into intracellular responses.

**splicing** Removal of introns from the precursor messenger RNA and joining of exons in mature messenger RNA; positions of boundaries between introns and exons are often conserved in homologous proteins of different species.

## Further Reading

Burtnick, L. D., Koepf, E. K., Grimes, J., Jones, E. Y., Stuart, D. I., McLaughlin, P. J., and Robinson, R. C. (1997). The crystal structure of plasma gelsolin: Implications for actin severing, capping and nucleation. *Cell* **90**, 661–670.

Choe, H., Burtnick, L. D., Mejillano, M., Yin, H. L., Robinson, R. C., and Choe, S. (2002). The calcium activation of gelsolin: Insights

from the 3Å structure of the G4–G6/actin complex. *J. Mol. Biol.* **324**, 691–702.

Concha, N. O., Head, J. F., Kaetzel, M. A., Dedman, J. R., and Seaton, B. A. (1993). Rat annexin V crystal structure: $Ca^{2+}$-induced conformational changes. *Science* **261**, 1321–1324.

Dos Remedios, C. G., Chhabra, D., Kekic, M., Dedova, I. V., Tsubakihara, M., Berry, D. A., and Nosworthy, N. J. (2003). Actin binding proteins: Regulation of cytoskeletal microfilaments. *Physiol. Rev.* **83**, 433–473.

Gerke, V., and Moss, S. E. (2002). Annexins: from structure to function. *Physiol. Rev.* **82**, 331–371.

Huber, R., Römisch, J., and Paques, E. P. (1990). The crystal and molecular structure of human annexin V, an anticoagulant calcium, membrane binding protein. *EMBO J.* **9**, 3867–3874.

Pallanck, L. (2003). A tale of two $C_2$-domains. *TINS* **26**, 2–4.

Rizo, J., and Südhof, T. C. (1998). $C_2$-domains, structure and function of a universal $Ca^{2+}$-binding domain. *J. Biol. Chem.* **273**, 15879–15882.

Swairjo, M. A., and Seaton, B. A. (1994). Annexin structure and membrane interactions: A molecular perspective. *Annu. Rev. Biophys. Biomol. Struct.* **23**, 193–213.

## BIOGRAPHY

Joachim Krebs has been working in the field of calcium-binding and calcium-transporting proteins for many years. After receiving his Ph.D. from the University of Tuebingen, Germany, he spent 2 years as a postdoctoral fellow at the Institute of Inorganic Chemistry of the University of Oxford, UK, before joining the Institute of Biochemistry at the Swiss Federal Institute of Technology (ETH) in Zurich, Switzerland. He has authored, co-authored, and edited numerous articles in international journals and books in the field of calcium biochemistry.

# Calcium-Modulated Proteins (EF-Hand)

**Robert H. Kretsinger**
*University of Virginia, Charlottesville, Virginia, USA*

**Many different proteins – extracellular, membrane, and intracellular – bind calcium more or less selectively. Those calcium binding proteins in the cytosol or bound to membranes facing the cytosol are inferred to be calcium modulated. That is, when the cell is quiescent, the concentration of free calcium ion is less than $10^{-7}$ M (pCa > 7) and the calcium-modulated protein is in the apo- or magnesi-form. Following stimulus, the concentration of calcium rises (pCa < 5.5), and the protein binds calcium. The attendant change in structure is involved in the transduction of the information of a pulse or wave of $Ca^{2+}$ ions to an ultimate target enzyme or structure. Most of these calcium-modulated proteins contain from 2 to 12 copies of the EF-hand domain. There are other proteins in the cytosol that bind calcium and appear to be modulated by calcium; these include the annexins, proteins that contain one or several C2 domains, such as protein kinases C or synaptotagmin, and calcium pumps.**

## Calcium Coordination

Many of the functional characteristics of calcium and of calcium-modulated proteins can be rationalized from the geometry of calcium coordination.

### Pentagonal Bipyramid

In proteins, the $Ca^{2+}$ ion (atomic radius 0.99Å) is usually bound by seven oxygen atoms in an approximately pentagonal bipyramid conformation at average Ca–O distance 2.3 ± 0.3Å; the oxygen atoms have some lateral flexibility. The $Mg^{2+}$ ion (atomic radius 0.65Å) is usually coordinated by six oxygen atoms at the vertices of an octahedron with Mg–O, 2.0Å; these oxygens are in tight van der Waals contact with one another. Although many small molecules bind magnesium with greater affinity than they bind calcium, most intra- and extra-cellular proteins bind calcium with much greater affinity than they bind magnesium.

### Kinetics of Calcium and Magnesium Binding

The dissociation constant is the ratio of the off rate to the on rate: $K_d$ (M) = $k_{off}$ ($s^{-1}$)/$k_{on}$ ($M^{-1}s^{-1}$). The rate limiting dehydration of $Ca(H_2O)_7^{2+}$ is fast, $\sim 10^{8.0}\ s^{-1}$; while that of $Mg(H_2O)_6^{2+}$ is slow, $\sim 10^{4.6}\ s^{-1}$. This reflects the loose pentagonal bipyramidal vs the tight octahedral packing of the oxygen ligands. The cation must be (partially) dehydrated before it can bind to the protein. These rates are extremely important for modeling the flux of $Ca^{2+}$ ions through the cytosol and the attendant binding of proteins. The increase in affinity of most proteins for calcium relative to magnesium derives primarily from this difference in $k_{on}$ (e.g., see Table I).

### Temporal Buffering

It is intriguing that most EF-hand proteins bind calcium ~10,000 times more strongly than they bind magnesium. This reflects strong evolutionary selection and cannot yet be mimicked by protein designers. The cytosolic concentration of the free $Ca^{2+}$ ion is $\sim 10^{-7.2}$ M and that of the $Mg^{2+}$ ion is $\sim 10^{-2.8}$; in contrast, both $pCa_{out}$ and $pMg_{out}$ are ~ 2.8. This means that a cytosolic protein, such as parvalbumin, that binds calcium with high affinity, e.g., $pK_d(Ca^{2+})$ ~8.0, will also bind magnesium with relatively high affinity, e.g., $pK_d(Mg^{2+})$ ~4.1, and, in the resting cell, will be in the magnesium state. In contrast, lower affinity sites, such as EF-hands 1&2 of troponin C have lower affinities for divalent cations, $pK_d(Ca^{2+}) \sim 6.5$ and $pK_d(Mg^{2+}) \sim 2.3$, and are apo in the resting state. This leads to the counterintuitive situation in which a pulse of messenger calcium first binds to the weaker apo sites in domains 1 and 2 of troponin C, misleadingly referred to as calcium specific. The $Ca^{2+}$ ion, whose concentration during the pulse reaches $10^{-6.0}$ M, can bind to the apo site only after the $Mg^{2+}$ ion diffuses off the strong site of parvalbumin. Parvalbumin, with higher affinity for divalent cations, binds calcium after the weaker

 © 2004, Elsevier Inc. All Rights Reserved. 

TABLE I

| | $K_d$ (M) | $k_{off}$ ($s^{-1}$) | $k_{on}$ ($M^{-1}s^{-1}$) |
|---|---|---|---|
| Parvalbumin/$Ca^{2+}$ | $10^{-8.0}$ | $10^{0.0}$ | $10^{8.0}$ |
| Parvalbumin/$Mg^{2+}$ | $10^{-4.1}$ | $10^{0.5}$ | $10^{4.6}$ |
| Troponin C (domains 1&2)/$Ca^{2+}$ | $10^{-6.5}$ | $10^{1.5}$ | $10^{8.0}$ |
| Troponin C (domains 1&2)/$Mg^{2+}$ | $10^{-2.3}$ | $10^{2.3}$ | $10^{4.6}$ |

troponin C and thereby helps to relax the muscle and prevent tetany. Such temporal buffering surely plays a significant role in the propagation and transduction of calcium waves observed in many cell types. The information encoded in the frequencies, durations, and amplitudes of calcium waves and spikes might be decoded by a corresponding matching of $k_{off}$ and $k_{on}$ rates for calcium and for magnesium in calcium-modulated proteins.

## EF-Hand Containing Proteins

The structure of the EF-hand provides insight into its evolution and function.

### Structure of the EF-Hand

The canonical EF-hand (Figure 1) consists of $\alpha$-helix E, (forefinger, residues 1–10), a loop around the $Ca^{2+}$ ion (clenched middle finger, 10–21), and $\alpha$-helix F (thumb, 19–29). Residue 1 is often Glu; the insides of the helices (palmer surfaces) usually have hydrophobic residues that contact the insides of the other EF-hand of the pair (Figure 2). The side chains of five residues, approximating the vertices of an octahedron (*X*, residue 10; *Y*, 12; *Z*, 14; $-X$, 18; and $-Z$ 21), provide oxygen atoms to coordinate $Ca^{2+}$; residue 16 at $-Y$ bonds to $Ca^{2+}$ with its carbonyl oxygen. The positions of these ligands within the loop are often referred to as 1, 3, 5, 7, 9, and 12. The $Ca^{2+}$ ion is actually 7 coordinate in a pentagonal bipyramid with major axis, *X*, $-X$. There are five oxygen atoms in the *Y*, *Z* plane; since, the $-Z$ ligand (residue 21, usually Glu) coordinates $Ca^{2+}$ with both oxygen atoms of its carboxylate group. A Gly at 15 permits a tight bend; residue 17 has a hydrophobic side chain that attaches the loop to the hydrophobic core of the pair of EF-hands.

Several variations to this canonical calcium coordination scheme have been inferred from amino acid sequence and confirmed in crystal structures of other EF-hand proteins. Nearly one-third of all known EF-hands do not bind calcium; those with no indels

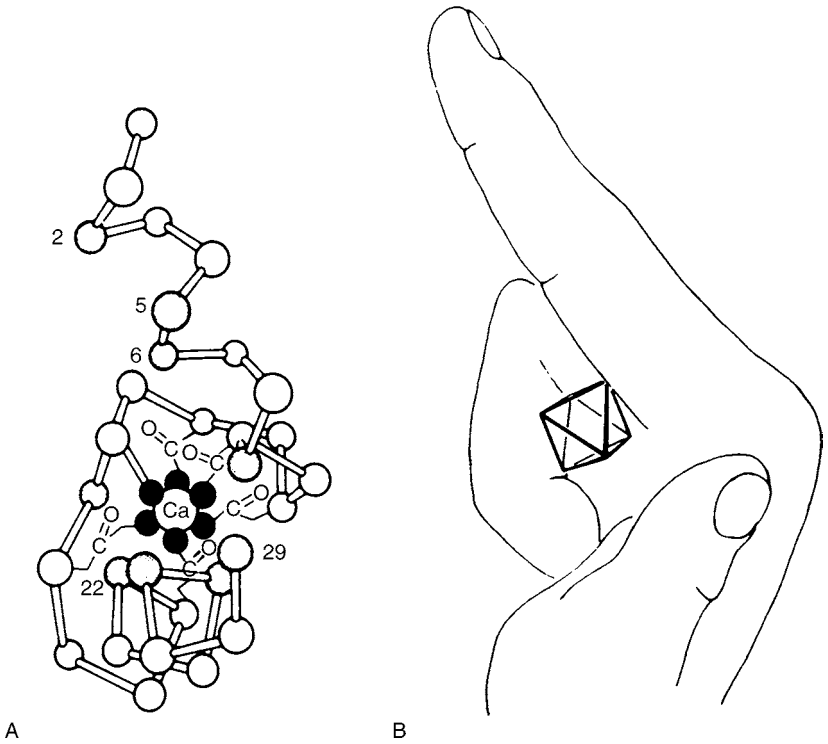



**FIGURE 1** The EF-hand. (A) The spheres represent $\alpha$-carbons. Residues 2, 5, 6, and 9 of helix E and residues 22, 25, 26, and 29 of helix F are usually hydrophobic and face the hydrophobic surface of the other EF-hand of the pair. Five residues—10, 12, 14, 18, and 21—coordinate the $Ca^{2+}$ ion with an oxygen from a side chain. Residue 21 is usually Glu and contributes both oxygen atoms of its carboxylate to the pentagonal bipyramid coordination of calcium. Residue 16 coordinates with its carbonyl oxygen. (B) Helix E, the loop around the $Ca^{2+}$ ion, and helix F are represented by the forefinger, clenched middle finger, and thumb.

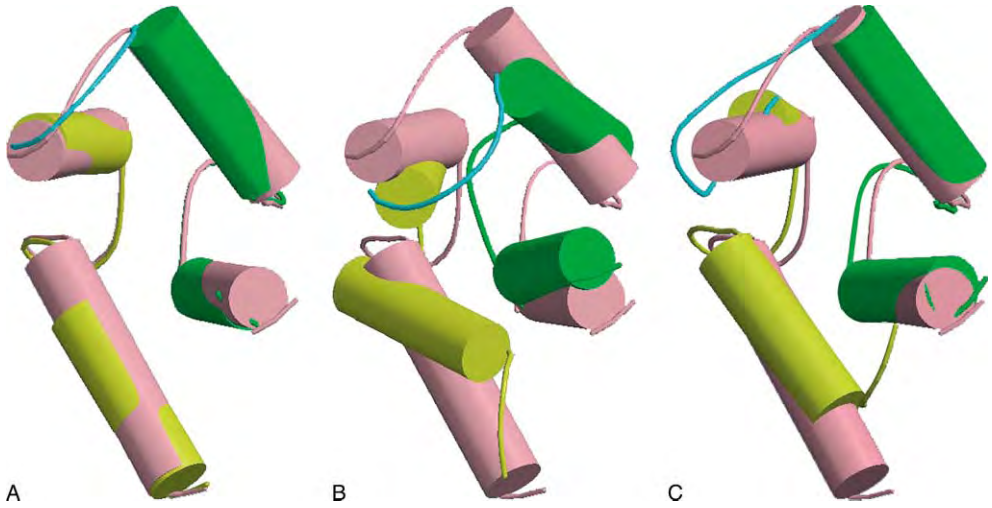



**FIGURE 2** Conformations of pairs of EF-hands. Calmodulin consists of two pairs (1&2 and 3&4) of EF-hands. A flexible tether, seven residues long, connects helix F2 to helix E3. In the crystal structure, F2, the tether, and E3 form a single continuous helix. Calmodulin is widely distributed and interacts with at least 30 different target proteins. Furthermore, calmodulin's four EF-hands are all canonical; hence, it serves as a reference point for many evaluations and comparisons. (A) The backbone of the pair calmodulin-3&4 in its dicalci form is shown as a cylinder when it is $\alpha$-helical and as a strand elsewhere. It is viewed down its approximate twofold axis and is shown in light salmon color; the calcium binding loops are far from the viewer. Dicalci-parvalbumin-CD&EF (CD, yellow; linker, light blue; EF, green) is superimposed on dicalci-calmodulin-3&4; their conformations are very similar. (B) Apo-calmodulin-3&4 (yellow, blue, green) is superimposed on dicalci-calmodulin-3&4 (salmon); the binding of calcium opens the cleft between the two helices of each EF-hand and between the two EF-hands of the pair. This is supposedly a necessary step permitting calmodulin to interact with its numerous targets. The structure of apo-parvalbumin is not known. (C) Dicalci-calmodulin-3&4 (yellow, light blue, and green) as complexed with the target peptide from myosin light chain kinase (not shown) is superimposed on dicalci-calmodulin-3&4 (salmon). Both the 3&4 and the 1&2 pairs of calmodulin undergo little additional change in conformation to bind their targets. The binding of a target peptide facilitates binding of calcium and vice versa. (Courtesy of Hiroshi Kawasaki, Yokohama City University, Yokohama, Japan.)

(insertions or deletions), have a non-oxygen-containing side chain substituted at position 10, 12, 14, 18, or 21. Other EF-hands have indels; most notable is EF-hand 1 of the S-100 subfamily (Table II), in which several carbonyl oxygens, instead of side chain oxygens, coordinate $Ca^{2+}$.

## Evolution of the EF-Hand Family

All members of a protein homolog family are inferred to have evolved from a common precursor domain in a single ancestral organism. The most parsimonious interpretation is that all of the EF-hand domains listed in Table II are homologs; however, the statistics are weak since the domains are only 30 residues long. We employ other criteria, such as the fact that nearly all EF-hands occur in pairs (Figure 2) and that in nearly all EF-hand proteins at least one of the EF-hands binds calcium. Many domains have been suggested to resemble the EF-hand. They are not included in Table II unless they have passed a Hidden Markov Model test based on unambiguous EF-hands of known structure. Most EF-hand containing proteins have been found in eukaryotes; however, there are several examples in eubacteria. EF-hand proteins, e.g., calmodulin, have been found in all eukaryotes subject to thorough investigation. This distribution might reflect an origin in the bacterium that gave rise to eukaryotic cells; other bacteria may have lost their EF-hands. Or, the precursor EF-hand may have arisen in an early eukaryote and been transferred to a few bacteria by some sort of transduction or transformation.

## Distribution of EF-Hand Domains

Most of the calcium-modulated proteins contain 2 to 12 tandem copies of the EF-hand domain. EF-hands occur in pairs and are related by an approximate twofold axis of rotation (Figure 2). Although about one-third of all EF-hands are known or inferred not to bind calcium, usually at least one EF-hand domain in any protein does bind calcium with $pK_d(Ca^{2+}) \sim 7.0$. The protein, such as archetypical calmodulin, is in the apo or magnesium form prior to stimulation of the cell; following a rise in $[Ca^{2+}]_{cyt}$, the competent EF-hand binds calcium with attendant change in conformation of itself and probably of the paired EF-hand of the two-domain lobe. If the protein is hetero-chimeric with a non-EF-hand catalytic portion, the change in conformation of the EF-hand region activates the enzyme. If the EF-hand protein itself is not catalytic, the change in conformation causes the EF-hand protein to activate a target enzyme or structural protein.

The characteristics of 77 distinct EF-hand proteins are summarized in Table II. The functions of only 26 of

**TABLE II**

**EF-Hand Containing Proteins**

| Name | Animal Plant Fungi Protist | Func. | Struct. | 1 (7 | 2 8 | 3 9 | 4 10 | 5 11 | 6 12) |
|---|---|---|---|---|---|---|---|---|---|
| CTER | | | | | | | | | |
| CAM | calmodulin | APFP | +X | + | + | + | +/− | | |
| TNC | troponin C | A… | +X | +/− | + | +/− | + | | |
| ELC | essential light chain, myosin | A.F. | +X | a/− | +/− | +/− | +/− | | |
| RLC | regulatory light chain | A.FP | +X | + | − | − | − | | |
| TPNV | troponin, nonvertebrate | A… | +? | − | + | +/− | + | | |
| CLAT | CAM-like leaf (*Arabidopsis*) | P.. | ?? | + | + | + | +**** | | |
| SQUD | squidulin (*Loligo*) | A… | +? | + | + | + | + | | |
| CDC | CDC31 & caltractin | APFP | ?? | + | +/− | +/− | + | | |
| CAL | cal1 (*Caenorhabditis*) | A… | ?? | + | + | + | + | | |
| CAST | CAST | .P.. | ?? | ***+ | − | + | + | | |
| CPV | | | | | | | | | |
| CLNB | calcineurin B | A.F. | +X | + | + | + | + | | |
| P22 | p22 | A… | ?? | + | − | + | + | | |
| VIS | visinin & recoverin | A… | +X | − | + | + | − | | |
| CALS | calsenilin (*Homo*) | A… | ?? | ***− | − | + | + | | |
| DREM | DRE antag. modul. (*Homo*) | A… | +? | ***−/? | +/? | + | + | | |
| CMPK | CAM dep prot kinase (*Lilium*) | .P.. | +? | ***+ | + | + | + | | |
| SOS3 | Ca sens homo (*Arabidopsis*) | .P.. | +? | − | − | − | − | | |
| Pairings | | | | | | | | | |
| RTC | reticulocalbin (*Mus*) | A… | ?? | + | ?/+ | ?/+ | + | + | + |
| SCF | DNA supercoil fact (*Bombyx*) | A… | ?? | ***+ | − | + | + | + | + |
| CALP | calpain | A… | +X | ***+ | + | − | − | − | |
| SORC | sorcin/grancyclin | A.F. | +X | ***+ | + | − | − | − | |
| S100 | S100 | A… | ?X | b/− | + | | | | |
| ICBP | intestinal Ca binding protein | A… | ?X | b/− | + | | | | |
| HYFL | trichohylin profilag | A… | ?? | b/? | +*** | | | | |
| DGK | diacylglycerol kinase | A… | +? | ***+ | +*** | | | | |
| NUBN | nucleobindin & NEFA | A… | ?? | ***+ | +*** | | | | |
| CRGP | CAM rel gene product (*Homo*) | A… | ?? | + | + | | | | |
| ACTN | α-actinin | A.F. | +X | ***+/− | +/− | | | | |
| FDRN | α-spectrin & α-fodrin | A… | +? | ***− | − | | | | |
| GPD | Glycerol-P-dehydrogenase | A… | +? | ***− | + | | | | |
| AIF1 | allograft inflammatory factor | A… | ?? | ***+ | +/? | | | | |
| BM40 | osteonectin, SPARK | A… | ?X | ***c | + | | | | |
| QR1 | QR1 & SC1 | A… | ?? | − | + | | | | |
| Self | | | | | | | | | |
| EF12 | Ca Binding prot of nematodes | A… | ?? | ***+ | + | + | − | + | + |
| | | | | + | + | + | + | + | + |
| LPS | *L. pictus* SPEC resembl prot | A… | ?? | + | + | + | + | + | + |
| | | | | + | − | | | | |
| CLBN | calbindin 28 kDa, calretinin | A… | ?? | + | +/− | + | + | + | − |
| EP15 | EP15 | A… | +X | ***− | −*** | − | +*** | + | −*** |
| TCBP | Tetrahymena CaBP | …P | ?? | − | + | + | + | | |
| P26 | p260lf (*Rana*) | A… | ?? | − | −*** | − | − | | |
| PLC | phopholipase C | A.F. | +X | ***− | − | − | −*** | | |
| CBP | CBP1, CBP2 (*Dictyostelium*) | ..F. | ?? | + | + | + | + | | |
| Miscellaneous | | | | | | | | | |
| PFS | surface protein (*Plasmodium*) | …P | ?? | ***+ | + | + | + | + | −*** |
| CLSM | calsymin | bact | ?? | + | − | + | − | + | − |

*continues*

**TABLE II**

*Continued*

| Name | Animal Plant Fungi Protist | Func. | Struct. | 1 (7 | 2 8 | 3 9 | 4 10 | 5 11 | 6 12) |
|---|---|---|---|---|---|---|---|---|---|
| UEBP | URE3-BP | A… | +? | + | + | + | −? | −? | |
| CDPK | Ca dependent protein kinase | .P.. | +? | *** + | + | + | + | | |
| PFPK | protein kinase (*Plasmodium*) | …P | +? | *** + | + | + | + | | |
| SPEC | *Strongylocentrotus* CaBP | A… | ?? | +/− | + | + | +/− | | |
| TPP | p24 thyroid protein | A… | ?? | + | + | + | ? | | |
| 1F8 | 1F8 & TB17 & calflagin | …P | ?? | + | + | + | + | | |
| SARC | sarcoplasm Ca bind prot | A… | ?X | + | +/− | + | +/− | | |
| AEQ | aequorin & luciferin BP | A… | +X | + | *−* | + | + | | |
| PPTS | protein phosphatase | A… | +? | *** − | *−* | + | + | | |
| H32 | HRA32 (*Phaseolus*) | .P.. | ?? | + | + | + | + | | |
| EFH5 | EFH5 | …P | ?? | − | +/− | +/− | − | | |
| CVP | Ca vector prot (*Branch.*) | A… | ?X | − | − | + | + | | |
| PMAT | memb. assoc. (*Arabidopsis*) | .P.. | ?? | *** + | + | + | + | | |
| LAV | LAV1 (*Physarum*) | ..F. | ?? | *** + | + | + | + | | |
| CMSE | CaBP (*Saccharopolyspora*) | bact | ?? | +/− | +/− | +/− | +/− | | |
| MSV | MSV097 (*Entomopoxvirinae*) | virus | ?? | + | + | − | – | | |
| PARV | parvalbumin | A… | ?X | del | − | + | + | | |
| BCBP | brain calcium binding protein | A… | ?? | +/− | − | + | + | | |
| CSCJ | S. coelicolor CBP | bact | ?? | + | + | − | + | | |
| DYSN | dystrophin | A… | +X | *** − | − | − | − | | |
| FIMB | fimbrin | A.F. | +? | +/− | +/−*** | | | | |
| GRP | ras guan releasing prot (*Rattus*) | A… | ?? | *** +/? | +/?*** | | | | |
| PKD | PKD2L/polycystin | A… | ?? | *** − | +*** | | | | |
| RYR | ryanodine receptor/Ca release | A… | +? | *** − | +*** | | | | |
| CBL | proto-oncogene Cbl | A… | +X | *** − | d*** | | | | |
| CIB | Ca & integrin binding protein | A… | ?? | *** + | + | | | | |
| SENS | calsensin (*Haemopsis*) | A… | ?? | + | + | | | | |
| GRV | groovin (*Drosophila*) | A… | ?? | + | + | | | | |
| BET4 | calcium bind. pollen allergen | .P.. | ?X | + | + | | | | |
| CSCD | S. coelicolor CBP | bact | ?? | + | + | | | | |
| CBCC | C. crescentus CBP | bact | ?? | + | + | | | | |
| ACHE | acetylcholine esterase | A… | +X | *** − | − | | | | |
| NCAB | neuronal CaBP | A… | ?? | + | −*** | | | | |
| SWPN | swiprosin | A… | ?? | *** − | +*** | | | | |

*Note*. The 77 known EF-hand homolog subfamilies are described in several groups: CTER, those that are congruent with calmodulin, troponin C, essential, and regulatory light chains; CPV, those that are congruent with calcineurin B, p22, and visinin (recoverin); Pairings, those closely related between or among themselves but not closely related to other subfamilies; Self, those (some of) whose EF-hands are most closely related to other EF-hands within the same subfamily; and Miscellaneous, those whose domains do not show a strong and consistent pattern of similarity with other EF-hand subfamilies. The first domain of (pre)parvalbumin is inferred to have been deleted; hence, its domains are listed as 2(AB), 3(CD), and 4(EF). In congruent subfamilies, all of the EF-hands 1 (or *n*) resemble one another more closely than they resemble other EF-hands within their own protein. For 26 subfamilies, only one sequence is available; this is indicated by inclusion of genus name in parentheses or as part of the name of that subfamily. APFP refers to whether the protein is found in Animals, Plants, Fungi, and/or Protists. Five subfamilies are found in prokaryotes (bact) and one in a virus. The Func/Struct columns indicate whether a function is known + or not ? and whether a crystal structure is available X or not ?. The symbol *** before the first EF-hand column or after the last column indicates a protein with the non-EF-hand domain(s) to either the N-side or C-side of the EF-hands. For P26 and for EP15 *** indicates another domain between EF-hands 1 & 2 and EF-hands 3 & 4. Thirty-four, including the nine enzymes, of the 77 subfamilies are hetero-chimeric. For AEQ and PPTS, **−*** indicates that domain is not recognizable as an EF-hand by analysis of its sequence; however, its proximity to an otherwise unpaired EF-hand suggests that it may be an EF-hand and is inferred not to bind calcium. For each EF-hand is indicated whether calcium binding is observed (or inferred from sequence) + or not −. For some EF-hands there are instances of both binding and not binding calcium +/−. There are four examples—a, b, c, and d—of noncanonical EF-hand loops that bind calcium. Some loops inferred not to bind calcium may provide additional examples of noncanonical calcium binding loops. EF12 has 12 and LPS has 8 EF-hands; for ease of formatting EF-hands, 7 and on are listed under EF-hands 1 and on. Of the 77 distinct EF-hand proteins 56 have been found in animals. The functions of only 27 of the 77 are known. Nine of these are enzymes and have been demonstrated or inferred to be activated by the binding of calcium. Many, but certainly not all, of the remaining 50 function in the information transduction pathway summarized for calcium modulated proteins, such as calmodulin.

the 77 are known. Nine of these are enzymes and have been demonstrated or inferred to be activated by the binding of calcium. Many, but certainly not all, of the remaining 51 function in information transduction pathways as summarized for calcium-modulated proteins, such as calmodulin. However, others such as intestinal calcium binding protein, probably facilitate the diffusion of calcium through the cytosol; parvalbumin appears to function as a temporal buffer. Thirty-one, including the nine enzymes, of the 77 subfamilies are hetero-chimeric. In addition to their EF-hands, they contain other domains of different evolutionary origin and conformation. It is not unusual for a basic protein domain to find many uses, often spliced together with other domains; however, the EF-hand is one of the most widely distributed domains in eukaryotes, perhaps reflecting the range and subtlety of calcium signaling. The downstream regulation element antagonist modulator (DREAM) upon binding calcium dissociates from a DNA binding regulatory element that otherwise functions as a gene silencer. Whether this might provide a precedent for long-term potentiation remains to be seen.

## Calmodulin

Calmodulin is probably present in all cells of all eukaryotes. It consists of four EF-hands and is highly conserved in amino acid sequence. The second (F2) $\alpha$-helix of domain 2, the eight residue linker between domains 2 and 3 and the first (E3) $\alpha$-helix of domain 3 form a single continuous $\alpha$-helix ~28 residues long, thereby giving calmodulin a dumbbell shape as seen both in the crystal and in solution. Upon binding calcium, the relative orientations of the four helices in both lobes change, thereby exposing hydrophobic regions and permitting the interaction of calmodulin with target enzymes and structural proteins. Over 30 calmodulin targets have been reported. The structures of calmodulin complexed with $\alpha$-helical regions of several targets, such as myosin light chain kinase (MLCK), reveal what appears to be a general pattern for the interactions of four domain EF-hand proteins, such as calmodulin, troponin C, and the essential and regulatory light chains of myosin, with their respective targets. MLCK is self-inhibited by its own peptide 796–813; limited proteolysis removes this peptide. The 1–795 MLCK is then constitutively active. *In vivo*, calmodulin binds calcium, undergoes a change in conformation, binds $\alpha$-helix 796–813 of MLCK, and thereby removes the self inhibition of MLCK. Calmodulin assumes a near spherical shape when complexed with its target, as opposed to the elongated dumbbell shape of uncomplexed calmodulin. The eight-residue linker is flexible, permitting calmodulin to assume a broad range of conformations. Among these conformations are some that grasp the MLCK target peptide; other conformations fit other targets.

## See Also the Following Articles

Calcium-Binding Proteins: Cytosolic (Annexins, Gelsolins, $C_2$-Domain Proteins) • Calcium Signaling: Motility (Actomyosin–Troponin System) • ER/SR Calcium Pump: Function • ER/SR Calcium Pump: Structure • Plasma-Membrane Calcium Pump: Structure and Function

## Glossary

**calcium-modulated protein** Calcium binding protein found in the cytosol whose structure changes with binding and release of $Ca^{2+}$ ions associated with a pulse of messenger calcium.

**calmodulin** Protein consisting of two pairs of EF-hands. It is found in nearly all cells of all eukaryotes and interacts with some 30 different target enzymes or structural proteins.

**EF-hand** Domain of 30 amino acids consisting of an $\alpha$-helix, a loop around a $Ca^{2+}$ ion, and a second $\alpha$-helix.

**transduction** Process of changing energy and/or information from one modality to another.

## Further Reading

Berridge, M. (2001). The versatility and complexity of calcium signalling. In *Novartis Foundation Symposium*, Vol. 239, pp. 52–67. Novartis Foundation, London.

Gerke, V., and Moss, S. E. (2002). Annexins: From structure to function. *Physiol. Rev.* **82**, 331–371.

Nakayama, S., Kawasaki, H., and Kretsinger, R. H. (2000). Evolution of EF-hand proteins. *Topics Biol. Inorg. Chem.* **3**, 29–58.

Schuster, S., Marhl, M., and Hofer, T. (2002). Modelling of simple and complex calcium oscillations—From single-cell responses to intercellular signalling. *Eur. J. Biochem.* **269**, 1333–1355.

Sudhof, T. C. (2002). Synaptotagmins: Why so many? *J. Biol. Chem.* **277**, 7629–7632.

Toyoshima, C., and Nomura, H. (2002). Structural changes in the calcium pump accompanying the dissociation of calcium. *Nature* **418**, 605–611.

## Biography

Robert Kretsinger is Professor of Biology at the University of Virgina, Charlottesville, Virginia. He received his Ph.D. in biophysics from the Massachusetts Institute of Technology and carried out his post-doctoral training at the Medical Research Council Laboratory of Molecular Biology in Cambridge, England. He determined the first crystal structure of an EF-hand protein, parvalbumin, in 1970, and he has continued his research in various aspects of protein structure, evolution, and structure prediction.

# Calpain

**Hiroyuki Sorimachi and Yasuko Ono**
*University of Tokyo, Tokyo, Japan*

**Calpain (Clan CA, family C02, EC 3.4.22.17) is a ubiquitous intracellular $Ca^{2+}$-dependent cysteine protease that displays limited proteolytic activity at neutral pH. Calpain acts by proteolytically processing, rather than digesting, substrates to transform and modulate their structures and activities. Thus, calpain is viewed as a representative "modulator protease" that governs various cellular functions such as signal transduction and cell morphogenesis. Calpain belongs to the papain superfamily and constitutes one of the three distinct kingdoms, i.e., calpain-, papain-, and bleomycin-hydrolase-sub-superfamilies. The human genome has 14 genes that encode a calpain-like protease domain. These generate diverse types of calpain homologues possessing combinations of several functional domains such as a $Ca^{2+}$-binding domain (C2-type and EF-hand-type) and a Zn-finger domain. Furthermore, calpain homologues are increasingly being found in other organisms including insect, nematode, trypanosome, plant, fungus, yeast, and even some bacteria, thus constituting a superfamily possessing versatile functions. The importance of the physiological roles of calpains is reflected by the fact that particular defects in calpain functionality cause a variety of deficiencies in many different organisms. These include muscular dystrophies, diabetes and tumorigenesis in humans, embryonic lethality in mice, neurogenesis deficiency in flies, incomplete sex determination in nematodes, defects in aleurone cell development in maize, and alkaline/osmotic stress susceptibility in yeast.**

## History and Nomenclature

Calpain was described as early as 1964. After several "re-identifications," calpain, which was called CANP (calcium activated neutral protease) at that time, was finally purified into homogeneity in 1978. Both names, "calpain" and "CANP," were unified as calpain in 1991. In 1984, the cDNA for the catalytic subunit of calpain was cloned for the first time, revealing that calpain is a chimeric molecule consisting of a cysteine protease and a calmodulin-like $Ca^{2+}$-binding module. In the two decades following this event, hundreds of calpain-related molecules, including its endogenous specific inhibitor protein, calpastatin, have been identified through the use of cDNA cloning and genome/EST projects.

Two major ubiquitous calpains are found in mammals, $\mu$- and m-calpains. Since most of the studies carried out concerned $\mu$- and m-calpains, they are referred to as the "conventional" calpains. As the names suggest, $\mu$-calpain and m-calpain are activated by $\mu$M and mM levels of $Ca^{2+}$ *in vitro*, respectively. They are both hetero-dimers and consist of a common calpain small regulatory subunit (30K, ca. 28 kDa) and large distinct $\mu$- and m-calpain catalytic subunit ($\mu$CL and mCL, respectively, ca. 80 kDa), which have ca. 60% amino acid identity. Fourteen human calpain homologues have been numbered as in the case of caspases as shown in Table I and Figure 1. As an example, $\mu$CL is now called "calpain 1," i.e., $\mu$-calpain is a hetero-dimer consisting of a 30K subunit and calpain 1. To avoid unnecessary confusions, however, the original name and the gene product name are adopted in this section, e.g., $\mu$CL/CAPN1, mCL/CAPN2, p94/CAPN3, 30K/CAPNS1, hTRA-3/CAPN5.

## Structure and Function of Conventional Calpains

In general, potential substrates for calpain are very limited and specific. Most oligopeptides investigated are poor substrates for calpain. Although casein is not an *in vivo* substrate of calpain, it is a very good *in vitro* substrate and is used to assay calpain activity. Although the rules governing substrate specificity remain unclear, calpain is thought to recognize a large scope of 3D substrate structures rather than a particular primary amino acid sequence. For example, protein kinases, phosphatases, phospholipases, cytoskeletal proteins, membrane proteins, cytokines, transcription factors, lens proteins, and calmodulin-binding proteins are just some of the proteins suggested to be *in vivo* substrates. There has been no report suggesting differences in substrate specificity between $\mu$- and m-calpains.

Calpain has a very specific proteinaceous inhibitor *in vivo* called calpastatin. Calpastatin has four repeats of an inhibitor unit, each of which inhibits calpain. Both $\mu$- and m-calpains possess similar susceptibility

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Human Calpain Related Genes**[a]

| Gene | Chr. | Originally described names(s) of the gene product | Other name(s) used | Name in number | Domains | | | | Expression | Note |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Protease activity[b] | C2-like | 5EF-hands | C2 (T) | | |
| *The large subunits* | | | | | | | | | | |
| ***CAPN1*** | **11q12-13.1** | $\mu$CANP[c]/calpain-I large subunit | $\mu$-calpain large subunit($\mu$CL), $\mu$80K | Calpain 1 | + | + | + | − | ubiquitous | +30K/CAPNS1 = $\mu$-calpain |
| ***CAPN2*** | **1q32-41** | mCANP/calpain-II large subunit | m-calpain large subunit (mCL), m80K | Calpain 2 | + | + | + | − | ubiquitous except for mammalian erythrocytes | +30K/CAPNS1 = m-calpain |
| | | p94 | calpain 3, nCL-1 | Calpain 3a | + | + | + | − | skeletal muscle | binds to connectin/titin termination codon in the human |
| ***CAPN3*** | **15q15** | Lp82, Lp85, etc. | — | Calpain 3b, 3c etc. | + | + | + | − | lens, retina | first exon |
| ***CAPN5*** | **11q14** | calpain 5, hTRA-3 | nCL-3 | Calpain 5 | + | + | − | + | testis, brain | nematode TRA-3 homologue |
| ***CAPN6*** | **Xq23** | calpain 6 | calpamodulin, CANPX | Calpain 6 | − | + | − | + | placenta, embryonic muscles | nematode TRA-3 homologue, but no Cys at the active site |
| ***CAPN7*** | **3p24** | calpain 7, PalBH | — | Calpain 7 | n.d. | + | − | − | ubiquitous | Aspergillus PalB homologue |
| | | nCL-2 | — | Calpain 8a | + | + | + | − | stomach | |
| ***CAPN8*** | **1q32-41** | nCL-2′ | — | Calpain 8b | + | − | − | − | stomach | $Ca^{2+}$-dependent |
| ***CAPN9*** | **1q42.1-43** | nCL-4 | — | Calpain 9 | + | + | + | − | digestive tracts | 30K is required for activity |
| ***CAPN10*** | **2q37.3** | calpain 10a–h | — | Calpain 10a–h | n.d. | | | | ubiquitous | SNP is related to NIDDM |
| ***CAPN11*** | **6p12** | calpain 11 | — | Calpain 11 | n.d. | | | | testis | |
| ***CAPN12*** | **19q13** | calpain 12a–c | — | Calpain 12a–c | n.d. | + | + | − | hair follicle | |
| ***CAPN13*** | **2p21-22** | calpain 13 | — | Calpain 13 | n.d. | + | + | − | ubiquitous | mRNA not detected |
| ***CAPN14*** | **2p21-22** | calpain 14 | — | Calpain 14 n.d. | n.d. | + | − | − | n.d. | |
| ***CAPN15*** | **16p13.3** | SOLH | — | Calpain 15 | n.d. | − | − | − | ubiquitous | drosophila SOL homologue |
| *The small subunits* | | | | | | | | | | |
| ***CAPNS1*** | **19q13** | CANP/calpain small subunit | 30K, ccs1 | CAPNS1 | – | − | + | − | ubiquitous | regulatory subunit for $\mu$CL and mCL |
| ***CAPNS2*** | **16q13** | CAPNS2 | 30K-2, ccs2 | CAPNS2 | − | − | + | − | ubiquitous | intron less gene |
| *The calpain inhibitor* | | | | | | | | | | |
| ***CAST*** | **5q15-21** | CANP inhibitor/ calpastatin | — | – | − | − | − | − | ubiquitous | specific inhibitor for $\mu$-, m-calpains, and nCL-4 |

[a] Human disease and results of knock-out mice were taken together.

[b] (+) indicates that it was experimentally shown to have protease activity, and (−) in calpain 6 means that it has no active site cysteine residue.

[c] Abbreviations: n.d., not yet determined; CANP, $Ca^{2+}$-activated neutral protease; nCL, novel calpain large subunit; LGMD2A, limb-girdle muscular dystrophy type 2A; TRA-3, transform genotypic hermaphrodites; PalB, phosphatase mutants: loss in activity at alkaline pH but normal or increased activity at acidic pH; PalBH, PalB homologue; SNP, single nucleotide polymorphism; NIDDM, non-insulin-dependent diabetes mellitus; SOL, small optic lobes; SOLH, SOL homologue; ALG-2, apoptosis-linked gene-2; PDCD6, programmed cell death 6.

[d] The corresponding mammalian gene does not exist.

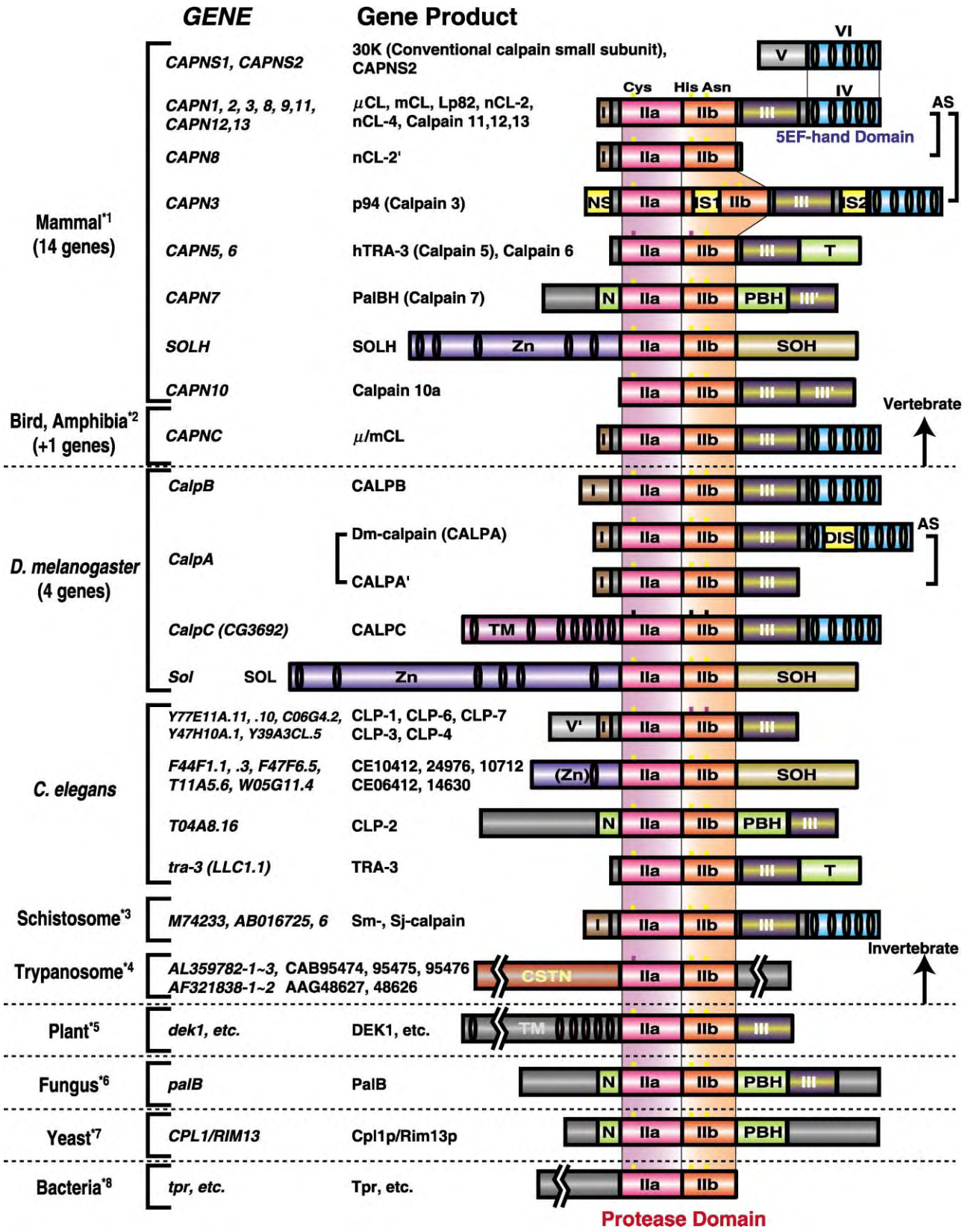



**FIGURE 1** Schematic structures of calpain. Calpain homologues have been identified in Mammals (*1: human, mouse, rat, rabbit, porcine, bovine, ovine), birds (*2: chicken and quail), amphibians (*2: *X. laevis*), flies, nematodes, schistosomes (*3: *S. mansoni* and *S. japonicum*), trypanosomes (*4: *Leishmania major* and *Trypanosoma brucei*), plants (*5: *A. thaliana*, maize, rice, and loblolly pine), fungi (*6: *A. oryzae* and *E. nidulans*), yeasts (*7: *S. cerevisiae*, *Y. lipolytica*, and *C. alabicans*) and bacteria (*8: *Porphyromonas gingivalis*, *Cyanobacterium Anabaena*, etc.). I,

to calpastatin. $\mu$- and m-calpains are ubiquitously expressed in mammalian and avian cells. Thus, their function is thought to be fundamental and essential. Many functions including the regulation of signal transduction systems, cell motility and apoptosis have been suggested, although they have not yet been completely clarified. *CAPN1* knock-out mice showed almost no phenotype, while *CAPNS1* knock-out mice resulted in embryonic lethality, indicating the indispensable roles of conventional calpains and, at the same time, differentiating between $\mu$- and m-calpain functionality.

As shown in Figure 1, the catalytic and regulatory subunits of conventional calpains can be divided into 4 and 2 domains, respectively. The N-terminus of domain I of the large subunit is autolyzed upon activation by $Ca^{2+}$. This results in a lower requirement for $Ca^{2+}$ and the dissociation of both subunits. Therefore, autolysis is involved in the regulation of calpain activity and specificity.

Three-D structural studies revealed that the protease domain in the absence of $Ca^{2+}$ is divided into two subdomains—domains IIa and IIb, which are folded into one domain upon $Ca^{2+}$ binding. This domain is highly conserved among calpain family members, suggesting the functional importance of this domain (Figure 1). Surprisingly, only the protease domain of $\mu$- and m-calpains showed $Ca^{2+}$-dependent protease activity. This is supported by the 3D structural studies of the protease domain in the presence of $Ca^{2+}$ that showed $Ca^{2+}$ bound to domains IIa and IIb. Thus, the whole calpain molecule mediates $Ca^{2+}$-dependency since all of the domains IIa, IIb, III, IV, and VI bind at least one $Ca^{2+}$ with varying affinities.

The 3D structure of domain III consists of 8 antiparallel $\beta$-strands ($\beta$-sandwich structure), a structure very similar to TNF-$\alpha$ and the C2-domains found in several $Ca^{2+}$-regulated proteins such as PKCs and synaptotagmins. Although the primary structure of domain III is highly conserved in calpain homologues, it has no similarity to any other proteins including TNF-$\alpha$ and C2-domains. This domain actually binds $Ca^{2+}$, and may play an important role in the $Ca^{2+}$-dependent membrane translocation of calpains.

Domain IV is very similar to domain VI of the small subunit, and each contains 5 EF-hand motifs. Thus, these domains are referred to as 5-EF-hand, or penta EF-hand (PEF) domains. *In vitro* experiments together with 3D structural studies showed that only the first, second, and third EF-hands bind $Ca^{2+}$. The fifth EF-hand motif is involved in the dimerization of both subunits.

Domain V of the calpain regulatory subunit contains clusters of Gly making it hydrophobic. This domain is thought to interact with membrane and/or membrane proteins through hydrophobic interactions. Most of this domain is cut off by autolysis, indicating no involvement of this region in protease activity. In humans, the *CAPNS2* gene encodes a regulatory subunit homologue, whose physiological roles remain unclear.

## Calpain Superfamily and Its Members

### Classifications

As for calpain homologues other than conventional calpains, domains other than the protease domain are not necessarily conserved amongst homologues (Figure 1). Amino acid sequence identities of the protease domains vary, depending on the molecules, from less than 30% to more than 75%. Several kinds of domains, putatively originated from independent genes, exist in both N- and C-terminal parts of the protease domain. These include C2 and C2-like domains, a 5-EF-hand domain, a transmembrane domain, as well as conserved domains with unknown functions (SOH, PBH, etc). These features, together with the organization of mammalian calpain genes, strongly suggest that calpain molecules are the result of the combination of the gene for the ancestral calpain-type cysteine protease with genes encoding other functions.

These calpain homologues can be divided into two categories. The first group consists of molecules having domains II, III, and IV (the protease domain), and the C2-like and 5-EF-hand $Ca^{2+}$-binding domains. In other words, these group members have a "typical" structure highly similar to conventional calpain catalytic subunits. Beside mammalian $\mu$CL/CAPN1 and mCL/CAPN2, typical calpains include p94/CAPN3, nCL-2/CAPN8, nCL-4/CAPN9, CAPN11, and CAPN12. Chicken and *Xenopus laevis* are reported to additionally have $\mu$/mCL. In invertebrates, only five typical calpains have been identified thus far. Three are found in *Drosophila melanogaster* as Dm-calpain (CALPA), CALPB, and CG-3692. *Schistosome mansoni* and

the N-terminal domain with little homology; IIa and IIb, the protease subdomains containing the active site Cys and His residues, respectively; III, the C2-like Ca2 + -binding domain; IV and VI, the 5-EF-hand Ca2 + -binding domain; V, Gly-rich hydrophobic domain; NS, IS1 and IS2, p94-specific sequences; T, TRA-3 subfamily-specific C2 domain; PBH, PalB subfamily homology domain; N, PalB subfamily N-terminal conserved domain; Zn, Zn-finger motif containing domain; SOH, SOL subfamily homology domain; DIS, CALPA-specific insertion sequence; TM, transmembrane domain; CSTN, the domain weakly similar to calpastatin.

*S. japonicum* also have at least one typical calpain (Sm-, and Sj-calpain). No typical calpain homologues have been found in *Caenorhabtisis elegans*, plants, fungi, trypanosomes, and *Saccharomyces cerevisiae*.

The second group contains various molecules that have the protease domain but do not have domains III or IV. Instead, some possess an extra domain(s) distinct from the known domains I to VI. Thus, these molecules are "atypical" calpain homologues. These atypical calpains are thought to have somewhat different functions compared with those of typical calpains. Atypical calpains include the TRA-3, SOL, and PalB subfamilies, the alternative splicing products of *Capn8* (nCL-2′) and *CalpA*, and others.

In addition to the structural features, independent classification is possible according to the localization of the expression. In mammals, μCL/CAPN1, mCL/CAPN2, PalBH/CAPN7, CAPN10, and CAPN13 are ubiquitously expressed, whereas p94/CAPN3, nCL-2/CAPN8, nCL-4/CAPN9, hTRA-3/CAPN5, CAPN6, 10, 11, and 12 are predominantly expressed in specific organs (Table I).

## Structure and Functions of Calpain Superfamily Members

### Skeletal Muscle-Specific Calpain, p94/CAPN3

p94/CANP3, the first tissue-specific calpain found in 1989, is ca. 60% identical to the large subunits of μ- and m-calpains, and has a conserved domain structure (Figure 1). Given that p94/CAPN3 contains three specific regions, NS, IS1, and IS2, it is often referred to as "calpain 3."

mRNA for p94/CAPN3 is expressed predominantly in skeletal muscle, and the amount expressed is approximately 10 times larger than that of conventional calpain. p94/CAPN3 possesses several unique properties. For example, p94/CAPN3 protein undergoes extremely rapid autolysis (half-life *in vitro* is less than 10 minutes), and this autolysis is obviated by deletion of the p94-specific region, IS1 or IS2. Specific inhibitors of μ- and m-calpains such as calpastatin, E-64, and leupeptin have no effect on autolysis. Furthermore, p94/CAPN3 possesses a nuclear localization signal-like sequence in IS2 and is localized in the nucleus in addition to the cytosol. p94/CAPN3 binds to gigantic muscle protein, connectin/titin, specifically through IS2. The protease activity of p94/CAPN3 should be regulated *in vivo*; however, the mechanism remains unclear. Connectin/titin is a candidate as a suppressor of p94/CAPN3 proteolytic activity.

Some alternative splicing products (Lp82) of *CAPN3* are specifically expressed in lenses. Lp82 showed $Ca^{2+}$-dependent protease activity against βA3 and αB crystallins. The activity is inhibited by E-64, but not by calpastatin. Some other splicing variants are expressed in embryonic skeletal muscles, although the physiological functions of these variants remain clear.

In 1995, mutations in *CAPN3* were shown to be responsible for limb-girdle muscular dystrophy type 2A (LGMD2A). Positions of the mutation found in the LGMD2A patients were distributed widely within *CAPN3*, with more than half of the mutations being missense mutations. No "hot point" was found, making its diagnosis very difficult. A primary cause of LGMD2A is a defect in protease activity, and not structural property, of p94.

### nCL-2 and nCL-2′ (CAPN8)

nCL-2 and nCL-2′ are alternative splicing products of *CAPN8*, with and without C2-like and 5-EF-hand domains, respectively. They are predominantly expressed in the stomach. nCL-2 is highly similar to mCL/CAPN2 along the whole molecule (ca. 62% identical). Moreover, *CAPN8* and *CAPN2* are closely located, and their transcripts have overlap, i.e., complementary sequences. Recombinant nCL-2 and nCL-2′ proteins were expressed in *E. coli* and showed $Ca^{2+}$-dependent caseinolytic activities. *X. laevis* possesses an nCL-2 orthologue, xCL-2, the disruption of which causes severe developmental defects.

### nCL-4/CAPN9

nCL-4/CAPN9 is a typical calpain homologue that is predominantly expressed in the digestive tract. It possesses overall similarity to μCL/CAPN1 and mCL/CAPN2, and requires 30K/CAPNS1 for its activity. Recombinant human nCL-4 + 30K protein showed $Ca^{2+}$-dependent caseinolytic activity, which was inhibited by calpastatin and other cysteine protease inhibitors, as in the case of conventional calpains. Involvement of nCL-4 in anti-tumorigenesis was reported in human gastric cancer and NIH3T3 transformation.

### TRA-3 and Its Orthologues (TRA-3 Subfamily)

TRA-3 is involved in the sex determination cascade of *C. elegans*. Although enzymatic characterization of the purified enzyme has not yet been reported, protease activity of TRA-3 is $Ca^{2+}$-dependent and necessary for female development in XX hermaphrodites through the processing of TRA-2A membrane protein. Mammals possess two orthologues of TRA-3, hTRA-3/CAPN5, and CAPN6, whose amino acid sequences are more than 30% identical to that of TRA-3. The T domain, which is conserved in all three molecules, has weak similarity to the C2-domain. Surprisingly, CAPN6 apparently has no

active site residues (active site Cys is substituted with Lys), strongly suggesting that CAPN6 has no proteolytic activity.

### Cpl1p, PalB, and its Orthologues (PalB Subfamily)

Cpl1p is the only calpain homologue found in *S. cerevisiae* and is considered both structurally and functionally to be the orthologue of Aspergillus PalB, which plays important roles in the adaptation of fungi to alkaline conditions. *CPL1*, also referred to as *RIM13*, is involved in both the alkaline adaptation and sporulation process of yeast through its processing activity. Rim101p and the Aspergillus orthologue PacC, are probable *in vivo* substrates for Cpl1p and PalB, respectively. Several other yeasts also have a Cpl1p orthologue. Cpl1p, PalB, and their orthologues share a somewhat conserved domain, the PalB homology domain (PBH). Mammals have one orthologue, PalBH/CAPN7, whose physiological functions are unknown.

### Calpain 10/CAPN10

This homologue was identified by reverse genetics of non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes). The single nucleotide polymorphism (SNP) in intron 3 of *CAPN10* is statistically related to a risk of NIDDM. This SNP probably affects transcription levels of *CAPN10*. *CAPN10* generates several alternative splicing products. The longest, CAPN10a, has two C2-like domains moderately and weakly similar to that of domain III. The physiological functions of calpain 10/CAPN10 are unclear.

### SOL and its Orthologues (SOL Subfamily)

The Drosophila gene responsible for a defect in neuronal cells (small optic lobes) was positionally cloned and shown to encode a calpain homologue with several Zn-finger motifs located at the N-terminus. Mammals possess one orthologue, SOLH/CAPN15, while *C. elegans* has several. They share a conserved C-terminal structure called the SOL homology domain (SOH).

### DEK1 and its Orthologues (DEK1 Subfamily)

The maize *defective kernel 1* gene required for aleurone cell development in the endosperm of maize grains revealed that it encodes a plant calpain homologue with 21 transmembrane regions located at the N-terminus and a C2-like domain, significantly similar to domain III, located at the C-terminus. Rice, Arabidopsis, and loblolly pine have very conserved orthologues (above 70% identity), and the whole genome sequence of Arabidopsis has revealed that DEK1 is the only calpain homologue in the genome. The structure and function of the DEK1 subfamily members are quite intriguing, and investigations have just begun.

### Other Calpain Homologues

As described with the TRA-3 subfamily, some of the calpain homologues possess substituted residues in one of the very conserved active site residues, Cys, His, and Asn. Besides CAPN6 and Drosophila CG3692, some of the *C. elegans* homologues, and all of the trypanosome homologues, do not possess one or more of the active site residues. These molecules are thought not to possess Cys protease activity. Some of the *C. elegans* calpain homologues possess Gly-rich sequences, which is a characteristic of domain V of the calpain regulatory subunits (CAPNS1 and CAPNS2). Trypanosome calpains have N-terminal domains weakly similar to calpastatin. The physiological significance of these structural features has yet to be determined.

## See Also the Following Articles

Calcium-Binding Proteins: Cytosolic (Annexins, Gelsolins, $C_2$-Domain Proteins) • Cysteine Proteases • Zinc Fingers

## Glossary

**C2 domain** Common $Ca^{2+}$-binding structure composed of 8 antiparallel $\beta$-strand structures.

**calpain homepage** Calpain-related reagents are now available from several companies, which are summarized on the calpain homepage (http://ag.arizona.edu/calpains/reagents.html).

**cysteine protease** Peptide bond hydrolyzing enzyme whose active site is composed of a catalytically active Cys residue.

**EF-hand motif** Common $Ca^{2+}$-binding motif composed of 2 $\alpha$-helices (E- and F-helices) and a $Ca^{2+}$-binding loop between.

**LGMD2A** Information on pathogenic mutations of LGMD2A is available in the Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/search/119751.html).

**muscular dystrophy** Progressive deterioration of muscle tissue and resultant weakness caused by a defect of the number of muscle genes such as dystrophin, sarcoglycan, merosin, laminin, and calpain.

## Further Reading

Glading, A., Lauffenburger, D. A., and Wells, A. (2002). Cutting to the chase: Calpain proteases in cell motility. *Trends Cell. Biol.* **12**, 46–54.

Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003). The calpain system. *Physiol. Rev.* **83**, 731–801.

Huang, Y., and Wang, K. K. (2001). The calpain family and human disease. *Trends Mol. Med.* **7**, 355–362.

Khorchid, A., and Ikura, M. (2002). How calpain is activated by calcium. *Nat. Struct. Biol.* **9**, 239–241.

Maki, M., Kitaura, Y., Satoh, H., Ohkouchi, S., and Shibata, H. (2002). Structures, functions and molecular evolution of the

penta-EF-hand $Ca^{2+}$-binding proteins. *Biochim. Biophys. Acta.* **1600**, 51–60.

Margis, R., and Margis-Pinheiro, M. (2003). Phytocalpains: Orthologous calcium-dependent cysteine proteinases. *Trends Plant Sci.* **8**, 58–62.

Ono, Y., Sorimachi, H., and Suzuki, K. (1999). New aspect of the research on limb-girdle muscular dystrophy 2A: A molecular biologic and biochemical approach to pathology. *Trends Cardiovasc. Med.* **9**, 114–118.

Sorimachi, H., and Suzuki, K. (2001). The structure of calpain. *J. Biochem.* **129**, 653–664.

Sorimachi, H., Ishiura, S., and Suzuki, K. (1997). Structure and physiological function of calpains. *Biochem. J.* **328**, 721–732.

Suzuki, K., Hata, S., Kawabata, Y., and Sorimachi, H. (2002). Structure activation, and biology of calpain. *Diabetes* **53**, S12–18.

## Biography

Hiroyuki Sorimachi is a Department Head of the Department of Enzymatic Regulation for Cell Functions, The Tokyo Metropolitan Institute of Medical Science (Rinshoken).

Yasuko Ono is a Chief Researcher in the Department of Enzymatic Regulation for Cell Functions, The Tokyo Metropolitan Institute of Medical Science (Rinshoken), devoting herself to the study of the role of calpains and the related molecules in muscle functions.

# Carbohydrate Chains: Enzymatic and Chemical Synthesis

**Thomas J. Tolbert and Chi-Huey Wong**
*Scripps Research Institute, La Jolla, California, USA*

**Carbohydrate chains play many important roles in biology, covering the surfaces of cells, mediating cell–cell recognition events, and forming major classes of biologically active molecules. Though carbohydrate chains are very important in biology, their study is frequently problematical because *in vivo* biosynthesis of carbohydrate chains often produces heterogeneous mixtures that are hard to purify in sufficient quantities for biochemical and structural studies. The *in vitro* enzymatic and chemical synthesis of carbohydrate chains offers another route to these interesting biomolecules that allows sufficient quantities of homogeneous oligosaccharides to be produced for biological studies.**

## Difficulties in the Synthesis of Carbohydrate Chains

The synthesis of oligosaccharides can present several technical difficulties that do not occur in the synthesis of other biopolymers. Unlike nucleic acids and proteins, the linkage between each subunit, i.e., sugar, of a carbohydrate chain forms a chiral center at the sugar's anomeric carbon that has two possible configurations, termed the $\alpha$- or $\beta$-anomer (Figure 1B). Each time a sugar is joined to a carbohydrate chain through a glycosidic linkage, the stereochemistry of the bond that is formed must be controlled or directed in some manner to insure that the correct anomer is produced. In addition, carbohydrate chains are not just simple linear chains as DNA and proteins are, but can also be branched chains with increased complexity. Glycosidic linkages can be formed through each hydroxyl of a sugar, and when multiple hydroxyls on a single sugar form glycosidic linkages, branched oligosaccharide structures result. Distinguishing between the different hydroxyl moieties on sugars is another difficulty of carbohydrate chain synthesis. Each sugar of a carbohydrate chain can have several hydroxyl moieties that are nearly equivalent in chemical reactivity, making them difficult to differentiate from one another and adding several steps to most chemical syntheses. To overcome these difficulties, several enzymatic and chemical methods have been developed.

## Enzymatic Synthesis of Oligosaccharides

Enzymes have frequently been employed to synthesize carbohydrate chains, because they offer a few technical advantages over traditional organic synthesis. Enzymes generally do not require protecting groups to select the correct chemical moiety on their substrates, and in carbohydrate chain synthesis this is a great advantage. The many nearly chemically equivalent hydroxyl moieties of sugars make selective chemical protection of sugar hydroxyls a laborious task. The use of enzymes in carbohydrate chain synthesis can eliminate the need for both selective hydroxyl protection before forming the glycosidic linkage, and removal of the protecting groups after forming the glycosidic linkage, greatly reducing the number of steps required for synthesis of an oligosaccharide. Another advantage of the use of enzymes in carbohydrate chain synthesis is the stereoselectivity of glycosidic bond-forming enzymes, which often produce a single anomer during the formation of a glycosidic bond. In contrast, chemical methods frequently produce mixtures of anomers during glycosidic bond formation, which must be purified from one another after formation of the glycosidic bond. Though enzymatic synthesis of oligosaccharides has many advantages, it is often limited by the availability of specific enzymes needed to form certain types of carbohydrate structures, but with the increased number of enzymes being discovered by genomic research this should improve in the future. Two classes of enzymes that have been used to form carbohydrate chains will be discussed further below, glycosidases and glycotransferases.

 © 2004, Elsevier Inc. All Rights Reserved. 

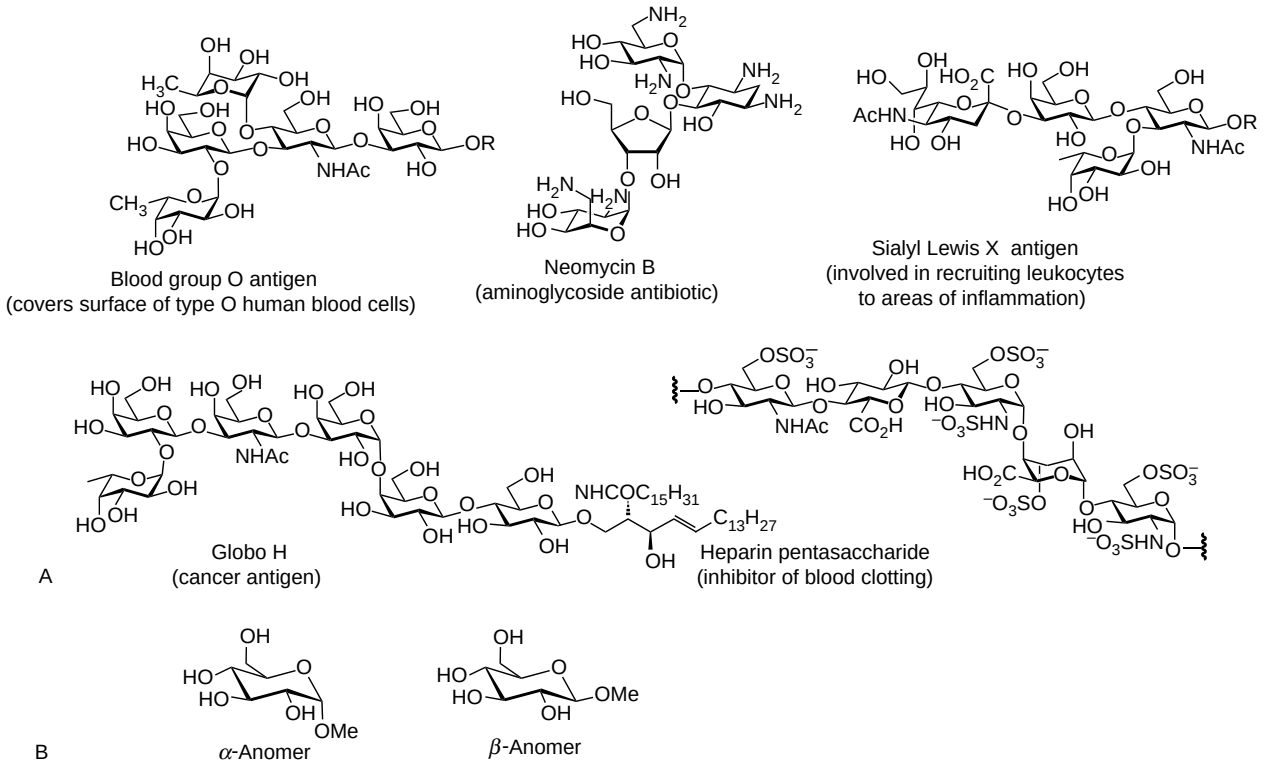



**FIGURE 1** Carbohydrate chains: (A) some biologically important oligosaccharides and (B) 1-O-methyl-glucopyranosides, $\alpha$- and $\beta$-anomers.

## Carbohydrate Chain Synthesis Utilizing Glycosidases

Glycosidases are enzymes that normally break glycosidic bonds during glycoprocessing or catabolism of oligosaccharides, but by placing glycosidases under certain controlled reaction conditions they can be utilized to form, rather than break, glycosidic bonds. Most often, glycosidases are used to form glycosidic bonds in transglycosylation reactions, where a glycosidic bond is broken in a glycosyl donor glycoside, and a new glycosidic bond is formed with a glycosyl acceptor (Figure 2A). Several approaches can be utilized to favor formation of the desired glycosidic bond, including the use of activated glycosyl donors such as *p*-nitrophenyl glycosides, elevated concentrations of glycosyl donors and acceptors, organic cosolvents, and the use of mutated glycosidases with reduced hydrolysis activity. The use of glycosidases to form glycosidic bonds generally results in low to medium yields ranging from 20% to 40%, although yields as high as 90% have been reported using mutated glycosidases. Glycosidase catalyzed synthesis generally has good stereoselectivity, forming a single $\alpha$- or $\beta$-anomer, but sometimes has low regioselectivity for the different hydroxyls on the acceptor sugar resulting in multiple products being formed. Though glycosidase reactions can suffer from low yield and low regioselectivity, there is a wide range of glycosidases available to catalyze the formation of many different types of glycosidic linkages.

## Carbohydrate Chain Synthesis Utilizing Glycotransferases

Glycotransferases are enzymes that catalyze the transfer of activated monosaccharide donors to carbohydrates during the biosynthesis of oligosaccharides. They are very useful in synthesizing oligosaccharides *in vitro* because they exhibit high regioselectivity and stereoselectivity in the formation of glycosidic bonds. Glycotransferases that utilize nucleotide sugars as activated monosaccharide donors have been used most often in the *in vitro* synthesis of carbohydrate chains (Figure 2B). A wide variety of oligosaccharide structures can be produced using the available glycotransferases and activated nucleotide sugars such as UDP-glucose (UDP-Glc), UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-galactose (UDP-Gal), UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-glucuronic acid (UDP-GlcUA), GDP-mannose (GDP-Man), GDP-fucose (GDP-Fuc), and CMP-sialic acid (CMP-NeuAc). Generally, each glycotransferase is selective for a specific

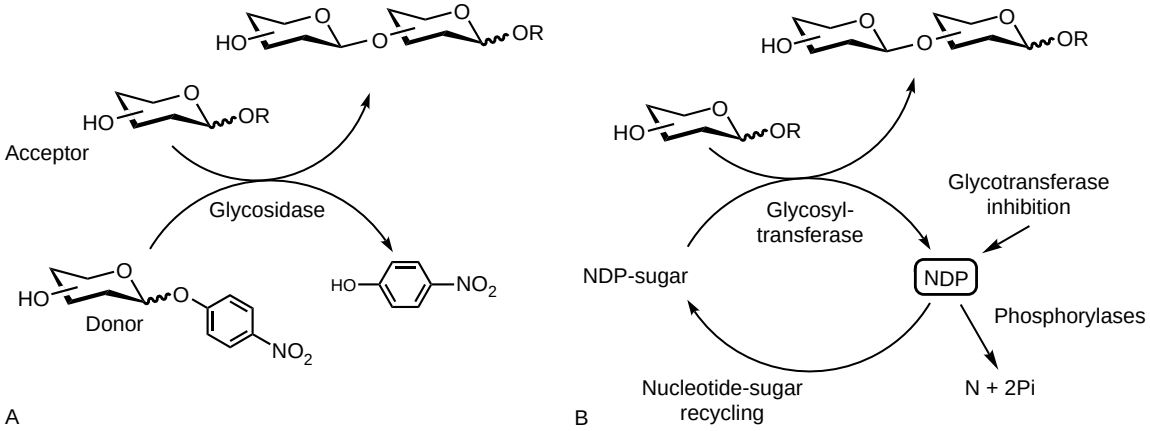



**FIGURE 2** Enzymatic synthesis of oligosaccharides using: (A) glycosidases and (B) glycotransferases.

nucleotide-sugar donor, and also has specificity for certain oligosaccharide structures in its substrates. Because of this it is often difficult to make unnatural oligosaccharides using glycotransferases, but in some cases relaxed substrate and nucleotide-sugar specificity has been utilized to make unnatural carbohydrate chains. Formation of oligosaccharides with glycotransferases requires the substrate oligosaccharide, manganese, the nucleotide-sugar donor, and the glycotransferase itself in an appropriate buffer. Glycotransferase-catalyzed synthesis of carbohydrate chains usually results in high yields, approaching 100%, but the enzymatic reaction can suffer from product inhibition that has to be overcome to achieve those high yields. The use of glycotransferases in oligosaccharide synthesis is limited by the high cost of nucleotide sugars and the availability of glycotransferases that form certain oligosaccharide structures. Sugar-nucleotide recycling alleviates some of the former problem while genomic sequencing and research has started to alleviate some of the latter problem.

### Product Inhibition in Glycotransferase Reactions

Though glycotransferases can be used to form oligosaccharides in nearly 100% yield, they suffer from product inhibition from the nucleoside diphosphates and nucleoside monophosphates that are produced as the activated nucleotide-sugar donors are transferred to the growing carbohydrate chain. This product inhibition can drastically slow the enzymatic reaction and also reduce the yield of product, and so it is desirable to remove the nucleotide by-products of the glycotransferase reactions. Two approaches can be used to overcome product inhibition in glycotransferase reactions use of phosphorylases and nucleotide-sugar recycling (Figure 2B).

***Reduction of Glycotransferase Inhibition with Phosphorylases*** Nucleoside diphosphates and monophosphates can be converted into nucleosides, which do not inhibit glycotransferases, using phosphorylases. This is a very simple method, which requires only one or two additional enzymes to be added to the glycotransferase reaction. Unfortunately this method requires a stoichiometric amount of nucleotide-sugar donor for formation of the desired oligosaccharide, which can be quite costly on larger scales since nucleotide sugars are generally very expensive.

***Reduction of Glycotransferase Inhibition with Nucleotide-Sugar Recycling*** Another method to remove nucleotide by-products of glycotransferase reactions is to regenerate the nucleotide-sugar donors from the nucleotide by-products using an enzymatic recycling reaction. This method of overcoming product inhibition is somewhat more complicated than using phosphorylases, requiring several additional enzymes for the nucleotide-sugar recycling reaction, but it allows a catalytic amount of nucleotide-sugar donor to be used in the glycotransferase reaction. Since nucleotide-sugars are generally expensive, this method is desirable for larger-scale glycotransferase reactions.

## Chemical Synthesis of Oligosaccharides

Chemical synthesis of oligosaccharides is often the method of choice for constructing carbohydrate chains because chemical methods are flexible, can be used to produce both natural and unnatural oligosaccharides, and are also not limited by the availability of specific enzymes. Formation of glycosidic bonds by chemical methods usually relies upon activation of a leaving group on the anomeric carbon of a glycosyl donor with a

Lewis acid, which once activated will react with a free hydroxyl upon a glycosyl acceptor (Figure 3A). A wide variety of glycosyl leaving groups have been utilized for carbohydrate chain synthesis some of which are shown in Figure 3B.

## Control of Stereochemistry in Chemical Glycosidic Bond Formation

Control of the stereochemistry of the anomeric linkage in the products of glycosylation reactions can be very complex, and many factors including types of protecting groups on the sugars, solvent, temperature, and leaving groups can be used to influence the $\alpha/\beta$ ratio of the products formed in glycosylation reactions. In general, the anomeric effect, stabilization of axial orientation over equatorial orientation of electron withdrawing groups attached to the C1 of pyranose sugars, can be utilized to produce $\alpha$-linked glucosides and galactosides. Participation of protecting groups of the 2-OH, such as acetate, and the use of polar solvents in glycosylation reactions can direct products toward $\beta$-linked glucosides and galactosides through dioxocarbenium or solvent intermediates (Figure 3A). Sometimes it is difficult to obtain the desired anomer using chemical synthesis, as in the case of sialic acid glycosides, where the natural sialic acid linkage is exclusively the $\alpha$-anomer, but the $\beta$-anomer is obtained from most chemical glycosylation reactions. Controlling the anomeric outcome of chemical glycosylation reactions can still be a difficult problem, and requires several approaches for different types of glycosidic linkages and sugars.

## Protection of Sugar Hydroxyls in Chemical Carbohydrate Synthesis

Chemical oligosaccharide synthesis relies heavily upon sugar protecting groups to both distinguish between sugar hydroxyls with similar chemical reactivity and also to control the stereochemical outcome, either $\alpha$ or $\beta$, of glycosylation reactions. Because of this, chemical oligosaccharide synthesis typically requires a large amount of protecting group manipulations. Normally, protecting groups must be placed on hydroxyls that are not going to be used to form glycosidic linkages to prevent them from reacting in glycosylation reactions. In addition, the synthesis of carbohydrate chains longer than two sugars requires sugar subunits that can act both as glycosyl donors and acceptors, and this usually requires selective protection and deprotection of the hydroxyls that are to form glycosidic linkages. Selective protection of sugar subunits for glycosylation reactions often requires many chemical steps to produce each selectively protected subunit for the synthesis of an oligosaccharide, and this is one factor that can contribute to the large number of steps required for chemical oligosaccharide syntheses. Some protecting groups commonly used in chemical carbohydrate chain synthesis include benzyl ethers, *p*-methoxybenzyl ethers, *tert*-butyl-diphenylsilyl ethers, *tert*-butyl-dimethylsilyl ethers, allyl ethers, acetate esters, benzoate esters, levulinoyl esters, and dimethyl acetals.

## Solution Phase Chemical Oligosaccharide Synthesis

In conventional solution-phase oligosaccharide synthesis, a donor sugar is activated by a Lewis acid at the anomeric carbon, and then reacted with an acceptor

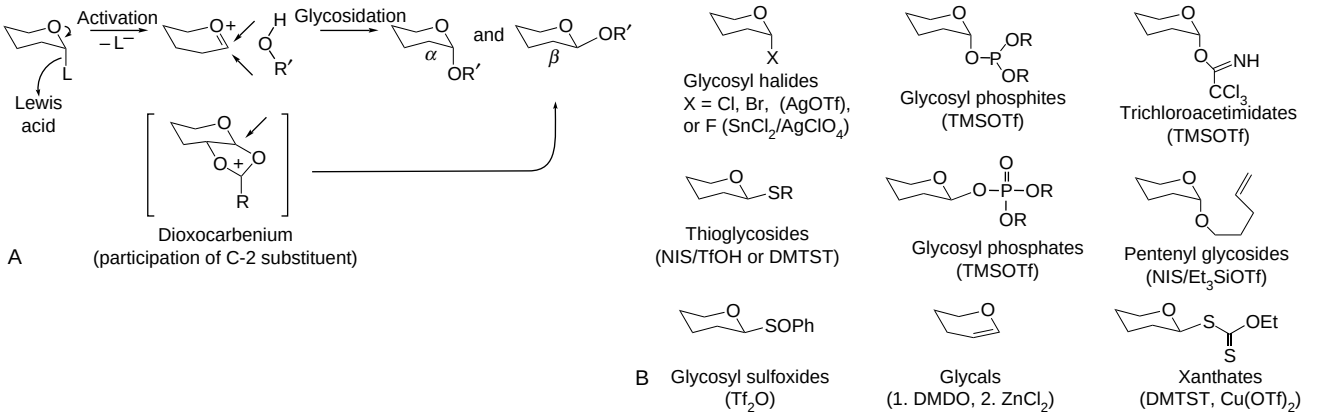



**FIGURE 3** Chemical glycosylation reactions. (A) Mechanism of glycosylation. (B) Some commonly used glycosylation leaving groups and the reagents used to activator them (in parentheses). Abbreviations: L, leaving group; R, variable group; TMSOTf, trimethylsilyl triflate; NIS, N-iodosuccinimide; TfOH, triflic acid; $Et_3SiOTf$, triethylsilyl triflate; $Tf_2O$, triflic anhydride; DMDO, 3,3-dimethyldioxirane; DMTST, dimethylthiosulfonium triflate.

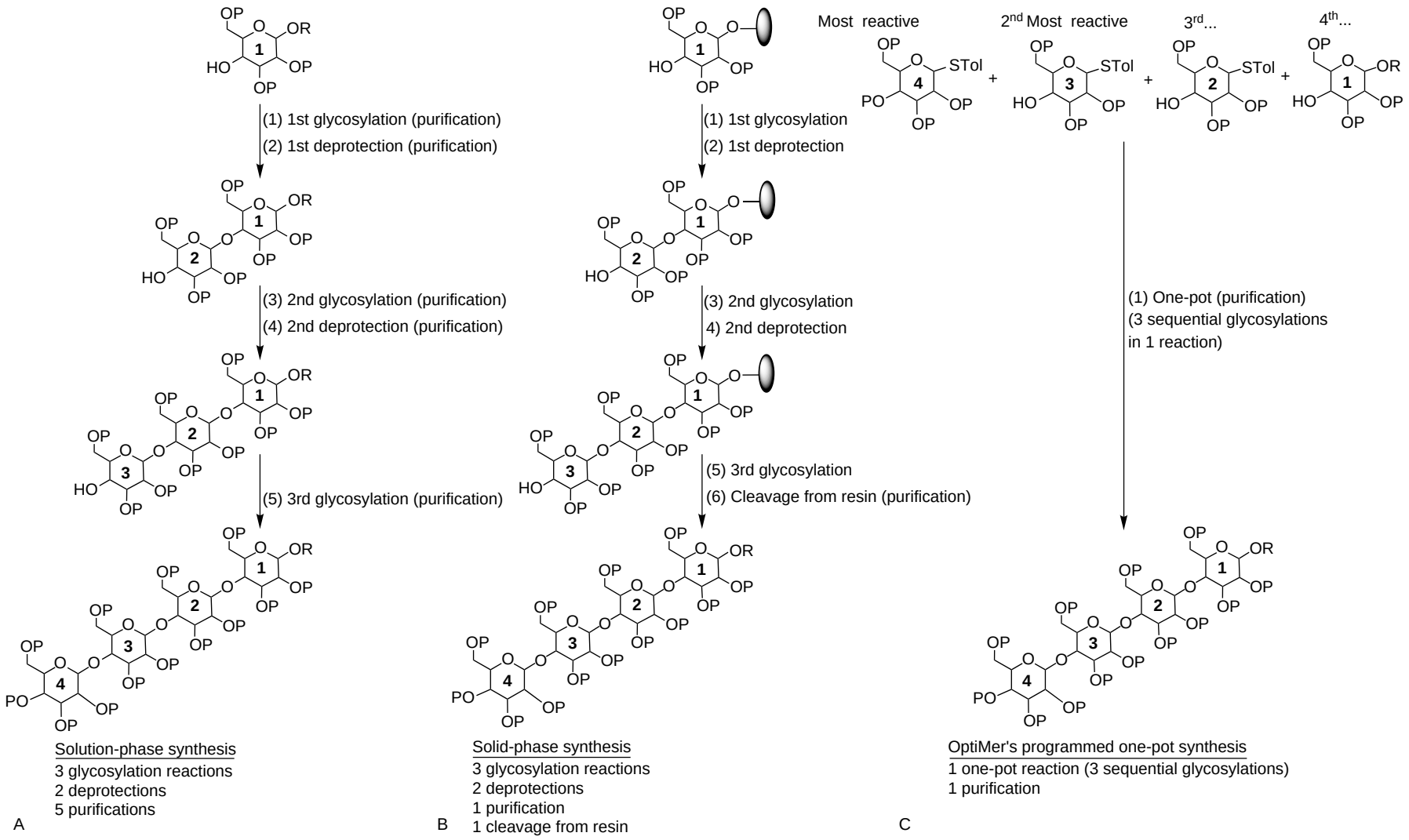



**FIGURE 4** Chemical synthesis of oligosaccharides: (A) solution-phase synthesis, (B) solid-phase synthesis, and (C) OptiMer's programmed one-pot synthesis.

sugar that contains a free hydroxyl. Once the glycosidic bond has been formed, the product disaccharide must be purified away from other glycosylation reaction reagents and side products, and then selectively deprotected to unmask a single hydroxyl so that it can then act as a glycosyl acceptor in the next glycosylation reaction (Figure 4A). Purification is often necessary after each glycosylation and deprotection step in the preparation of an oligosaccharide by solution phase synthesis. When combined with the number of steps necessary to produce selectively protected sugar subunits, the number of chemical steps and purifications necessary to construct even small oligosaccharides can be very large.

## Automated Oligosaccharide Synthesis

Because of the large amount of work necessary to construct even relatively small oligosaccharides by solution-phase synthesis, many approaches toward automating oligosaccharide synthesis have been developed in hopes of minimizing the number of chemical steps and purifications required to synthesize oligosaccharides. Two approaches to automated oligosaccharide synthesis will be discussed below: solid-phase oligosaccharide synthesis and programmable one-pot oligosaccharide synthesis.

### Solid-Phase Chemical Oligosaccharide Synthesis

In solid-phase oligosaccharide synthesis, either the glycosyl donor or acceptor of a glycosylation reaction is attached to the solid phase. The advantage of this is that large excesses of the other components of the glycosylation reaction can be used to increase the rate of the reaction and insure that it goes to completion, and then rinsed away by simple filtration. This has the potential of increasing the yield of glycosylation reactions and also eliminates many of the laborious purification steps that are necessary in solution-phase oligosaccharide synthesis (Figure 4B). Unfortunately there are many technical difficulties that must be overcome to successfully apply solid-phase synthesis to the production of oligosaccharides. Solid supports that function well with many solvents must be selected, since a wide variety of solvents are used to affect the outcome of glycosylation reactions. Linkers must be developed that are not sterically hindering for glycosylation reactions, stable to glycosylation conditions, and easily cleaved after the oligosaccharide has been synthesized. Strategies for protecting sugar monomers that allow highly flexible and selective deprotection of sugar hydroxyl groups in the presence of many other protected hydroxyls must be worked out. Since the reaction conditions of glycosidic bond formation can vary widely depending on oligosaccharide structure and the types of glycosidic linkages to be formed, there has been great difficulty in developing broadly applicable general methods for solid-phase oligosaccharide synthesis. Nevertheless, there have been many notable examples of successful solid-phase syntheses including the synthesis of a dodecasaccharide by both the Nicolaou and Seeberger groups.

### Programmable One-Pot Oligosaccharide Synthesis

One-pot reaction approaches that involve conducting several sequential glycosylation reactions in one reaction flask have been developed to facilitate automated oligosaccharide synthesis. The one-pot approach relies upon using a reactivity profile of protected sugars to determine what sequence of sugars to add to glycosylation reactions to obtain the desired products. Protected sugars are added to one-pot reactions in the order of most reactive to least reactive, thereby controlling the order of glycosidic bond formation during the synthesis of carbohydrate chains (Figure 4C). Since it has been shown that the types of protecting groups and type of anomeric activating groups used on sugars can greatly alter the speed at which a sugar will react in a glycosylation reaction, a wide range of reactivities can be obtained for a single type of sugar, allowing wide flexibility in oligosaccharide synthesis using this approach. The one-pot approach greatly reduces the number of steps necessary to construct oligosaccharides and eliminates several tedious purifications by combining multiple glycosylation reactions into a single one-pot reaction. The one-pot approach also minimizes protecting group manipulations, eliminating protecting group manipulations after construction of the building blocks. A large number of *p*-methylphenyl thioglycosides have been synthesized and had their reactivities measured to facilitate the use of this strategy. A computer program, OptiMer, has been developed that uses these *p*-methylphenyl thioglycosides as a set of building blocks to choose from to build oligosaccharides. When a carbohydrate chain structure is entered as input, the OptiMer program will calculate the best set of reactants for the construction of that carbohydrate chain. Because the programmable one-pot strategy requires a minimum of protecting group manipulations and has a large library of building blocks, a wide range of oligosaccharide structures can be synthesized rapidly using this method.

## See Also the Following Articles

Enzyme Reaction Mechanisms: Stereochemistry • Oligosaccharide Chains: Free, *N*-Linked, *O*-Linked

## Glossary

**anomeric carbon** The carbon of a cyclic sugar which forms a hemiacetal or hemiketal. In the linear form of the sugar, the carbon that will become the anomeric carbon when the sugar cyclizes is the carbonyl carbon.

**anomeric effect** The stabilization of axial orientation over equatorial orientation of electron withdrawing groups attached to the C1 of pyranose sugars.

**anomers** The pair of diastereomers, termed the $\alpha$- or $\beta$-anomer, that results when a linear sugar forms a cyclic hemiacetal or hemiketal.

**glycosidic linkage** The linkage formed between the anomeric carbon of a sugar and an alcohol.

## Further Reading

Koeller, K. M., and Wong, C.-H. (2000). Synthesis of complex carbohydrates and glycoconjugates: Enzyme-based and programmable one-pot strategies. *Chem. Rev.* **100**, 4465–4493.

Nicolaou, K. C., Watanabe, N., Li, J., Pastor, J., and Winssinger, N. (1998). Solid-phase synthesis of oligosaccharides: Construction of a dodecasaccharide. *Angew. Chem. Int. Ed.* **37**, 1559.

Plante, O. J., Palmacci, E. R., and Seeberger, P. H. (2001). Automated solid-phase synthesis of oligosaccharides. *Science* **291**, 1523.

Sears, P., and Wong, C.-H. (2001). Toward automated synthesis of oligosaccharides and glycoproteins. *Science* **291**, 2344–2350.

Seeberger, P. H., and Haase, W.-C. (2000). Solid-phase oligosaccharide synthesis and combinatorial carbohydrate libraries. *Chem. Rev.* **100**, 4349–4393.

Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., and Marth, J. (eds.) (1999). *Essentials of Glycobiology.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Wong, C.-H., Halcomb, R. L., Ichikawa, Y., and Kajimoto, T. (1995). Enzymes in organic synthesis: Application to the problems of carbohydrate recognition: Part 1. *Angew. Chem. Int. Ed.* **34**, 412–432.

Wong, C.-H., Halcomb, R. L., Ichikawa, Y., and Kajimoto, T. (1995). Enzymes in organic synthesis: Application to the problems of carbohydrate recognition: Part 2. *Angew. Chem. Int. Ed.* **34**, 521–546.

Zhang, Z., Ollman, I. R., Ye, X.-S., Wischnot, R., Baasov, T., and Wong, C.-H. (1999). Programmable one-pot digosaccharide synthesis. *J. Am. Chem. Soc.* **121**, 734.

## Biography

Thomas J. Tolbert is an Assistant Professor at Indiana University. His principal research interests lie in the use of chemical and enzymatic synthesis to solve biological problems. He holds a Ph.D. in Biochemistry from MIT, and a B.S. degree in chemistry from Purdue University.

Chi-Huey Wong is Professor and Ernest W. Hahn Chair in Chemistry at the Scripps Research Institute and also a Member of the Skaggs Insitute for Chemical Biology. His principal research interests are in the areas of bioorganic and synthetic chemistry and biocatalysis, with particular focus on development of new chemoenzymatic methods to tackle major problems in carbohydrate-mediated biological recognitions. He received his B.S. and M.S. degrees from National Taiwan University, and Ph.D. in Chemistry from Massachusetts Institute of Technology. He is a Member of the National Academy of Sciences.

# Carnitine and β-Oxidation

**Janos Kerner and Charles L. Hoppel**
*Case Western Reserve University School of Medicine, Louis Stokes VA Medical Center, Cleveland, Ohio, USA*

**Carnitine is derived from the essential amino acids lysine and methionine. It is ubiquitous in nature, found in especially high concentration in muscle tissue of higher organisms. It functions as a transport vehicle for activated fatty acids of different chain length through membranes within the cell. This transport function is best characterized in mitochondrial oxidation of long-chain fatty acids. This latter process, also known as mitochondrial β-oxidation, represents the repetitive oxidative cleavage of long-chain fatty acids into two-carbon units, acetyl-CoA. Acetyl-CoA is then either further oxidized for energy production (all tissues) or used to synthesize ketone bodies (liver) as metabolic fuel for peripheral tissues.**

## Carnitine Biosynthesis and Homeostasis

The first clue about the physiological function of carnitine (Figure 1) came from studies in the mealworm, *Tenebrio molitor*, that suggested that carnitine plays a vital function in fat catabolism. However, carnitine is not a vitamin for higher animals; the daily need is met by endogenous synthesis and dietary intake, mostly from meat products. For endogenous synthesis, the ultimate precursors are the essential amino acids lysine and methionine. Protein-bound lysine is methylated to trimethyllysine using S-adenosylmethionine; following liberation by proteolysis, the free trimethyllysine is converted (mostly in muscle) by a series of reactions to butyrobetaine, the ultimate carnitine precursor. Although most tissues are capable of synthesizing butyrobetaine, the hydroxylation of butyrobetaine to carnitine is restricted to the liver (and to a lesser extent to the kidney and brain). Following release of carnitine from the liver into the plasma, carnitine is taken up by other tissues in a carrier-mediated transport process (Figure 2).

The body distribution of carnitine is determined by a series of systems that transport carnitine into cells against a concentration gradient, an independent efflux process, and an exchange mechanism in a tissue-specific fashion. Under physiological conditions, plasma carnitine concentration is maintained within a narrow range, predominantly by renal clearance, which is determined by the kinetic properties of carnitine transport system in the brush border.

## Functions of Carnitine

In higher animals, the only reaction carnitine undergoes is a reversible transesterification with acyl-CoAs catalyzed by carnitine acyltransferases of differing chain length specificity (carnitine acetyl-, octanoyl-, and palmitoyltransferases) (Figure 3).

All known functions of carnitine are based on the reversible transesterification with acyl-CoA esters. The physiologically most important and therefore best studied metabolic pathway in which carnitine plays a significant role is the mitochondrial oxidation of long-chain fatty acids.

### Mitochondrial β-Oxidation

#### Cellular Uptake and Activation of Long-Chain Fatty Acids

Long-chain fatty acids represent a main energy source for many organs, especially for muscle (heart and skeletal) and liver. Since most tissues contain only small amounts of storage lipids, for energy production they depend on a continuous supply of fatty acids, mostly from adipose tissue. Following mobilization by lipolysis in adipose tissue and transport in the blood bound to albumin, fatty acids are taken up by the tissues in a process mediated by transport proteins present in the plasma membrane. Once within the cell, free fatty acids are bound to fatty acid binding proteins, which are present in the cytosol in large amounts. Depending on the tissue and its metabolic demand, fatty acids are converted to triglycerides and stored, secreted as very-low-density lipoproteins (liver), or oxidized in the mitochondria for energy production. Before being directed into storage or oxidation, fatty acids first are activated to acyl-CoA esters. This reaction is catalyzed by long-chain acyl-CoA synthetase, an enzyme present in abundance both in microsomes and in mitochondria. In mitochondria, long-chain acyl-CoA synthetase is

 © 2004, Elsevier Inc. All Rights Reserved. 

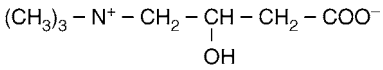



**FIGURE 1** Structure of L-carnitine (4-N-trimethylammonio-3-hydroxybutanoate).

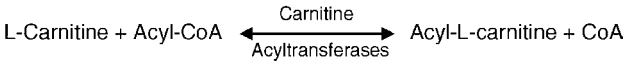



**FIGURE 3** Reversible transesterification of carnitine catalyzed by carnitine acyltransferases with different chain-lengths specificity.

associated with the mitochondrial outer membrane and appears to be a transmembrane protein with its active site exposed to the cytosol. This orientation of long-chain acyl-CoA synthetase gives rise to cytosolic production of long-chain acyl-CoAs, which for their further β-oxidation must cross both mitochondrial boundary membranes. Although the impermeability of the inner membrane to metabolites is well known, it is now recognized that the outer membrane is not freely permeable to solutes as previously thought. A potential route for long-chain acyl-CoAs to cross the outer membrane is the voltage-dependent, anion-selective channel (VDAC), also called mitochondrial porin, which occurs at high density in the mitochondrial outer membrane and regulates the permeability of this membrane to ions and metabolites (Figure 4).

### Mitochondrial Uptake of Activated Fatty Acids: The Mitochondrial Carnitine System

The mitochondrial carnitine system plays an obligatory role in β-oxidation of long-chain fatty acids by providing a means for their transport into the mitochondrial matrix. This transport system consists of the malonyl-CoA sensitive carnitine palmitoyltransferase-I (CPT-I) localized in the mitochondrial outer membrane, the carnitine:acylcarnitine translocase, an integral inner membrane protein, and carnitine palmitoyltransferase-II (CPT-II) localized on the matrix side of the inner membrane. In the schema depicted in Figure 4, the catalytic site of CPT-I faces the intermembrane space. In an alternative model, the catalytic site of CPT-I faces the cytosol. In this scenario, the carnitine esters must cross both mitochondrial membranes. In either case, transesterification of long-chain acyl-CoA esters to their carnitine esters by CPT-I commits activated fatty acids to oxidation in the mitochondrial matrix.

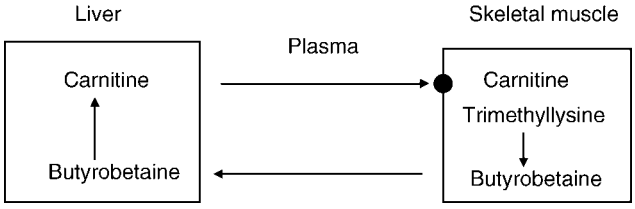



**FIGURE 2** Interorgan relationship in carnitine biosynthesis.

CPT-I, by virtue of its inhibition by malonyl-CoA, represents a key regulatory site controlling the flux through β-oxidation. Consistent with its central role in mitochondrial fatty acid oxidation, the enzyme exists in at least two isoforms with significantly different kinetic and regulatory properties. The liver type displays a higher affinity for carnitine and a lower affinity for the physiological inhibitor, malonyl-CoA, as opposed to the muscle isoform. Long-term regulation of both isoforms is accomplished at the transcriptional level. Short-term or acute regulation is achieved via changes in tissue malonyl-CoA concentration, and for the liver isoform also via changes in the enzyme's sensitivity to malonyl-CoA inhibition.

The long-chain acylcarnitines formed by CPT-I in the mitochondrial outer membrane enter the mitochondrial matrix in exchange for free carnitine catalyzed by the carnitine:acylcarnitine translocase, an integral inner membrane protein. The carnitine:acylcarnitine translocase catalyzes a homologous/heterologous carnitine exchange (in addition to the slow unidirectional carnitine transport) and displays increasing affinity for carnitine esters with increasing acyl chain length. Experimental data suggest the existence of a single transporter protein with a substrate specificity ranging

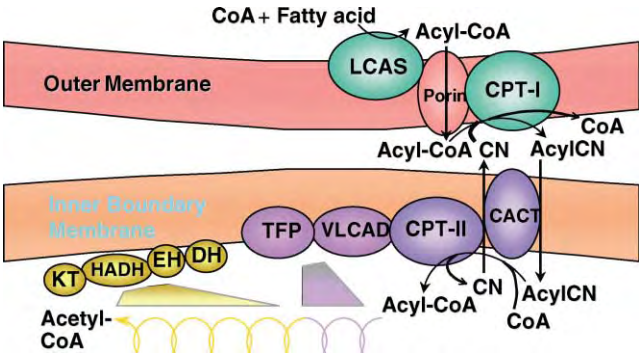



**FIGURE 4** Schematic depiction of the carnitine-dependent transport of activated fatty acids into the mitochondrial matrix and of their oxidative chain shortening via the β-oxidation pathway. CACT, carnitine:acylcarnitine translocase; CN, carnitine; CPT-I, malonyl-CoA sensitive carnitine palmitoyltransferase; CPT-II, malonyl-CoA insensitive carnitine palmitoyltransferase; DH, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HADH, 3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; LCAS, long-chain acyl-CoA synthetase; TFP, trifunctional protein; VLCAD, very-long-chain acyl-CoA dehydrogenase.

from free carnitine to long-chain carnitine esters. This is in contrast to the known substrate specificity of the mitochondrial carnitine acyltransferases (carnitine palmitoyl- and acetyltransferase) and implies that in addition to its role in mitochondrial fatty acid oxidation, the same transporter also is engaged in other functions involving membrane transport of acyl groups.

Following the translocation of long-chain acylcarnitines by carnitine:acylcarnitine translocase into the mitochondrial matrix, the carnitine esters are reconverted to their respective CoA esters by CPT-II, thus completing the carnitine-dependent uptake of activated fatty acids. Within the matrix, CPT-II is anchored to the inner membrane as a peripheral membrane protein. The enzyme has been isolated in catalytically active form from a variety of tissues, and its physical, kinetic, and immunological properties have been determined. CPT-II is the product of a single gene and is expressed as the same protein in all tissues within the body. It is not inhibited by malonyl-CoA, either in its native environment (membrane-bound) or when isolated.

### Pathway of Mitochondrial β-Oxidation

In the fatty acid oxidation cycle, the activated long-chain fatty acid undergoes a consecutive oxidative chain shortening. Each cycle of this sequence of four reactions results in the removal of two-carbon atoms from the fatty acyl residue in the form of acetyl-CoA (Figures 4 and 5) and two reducing equivalents (FADH and NADH). Thus, the complete oxidation of palmitoyl-CoA requires seven cycles, yielding eight acetyl-CoAs and seven FADH and NADH. Acetyl-CoA is then further oxidized in the tricarboxylic acid cycle to $CO_2$ and reducing equivalents, each acetyl-CoA yielding three NADH and one FADH. The reoxidation of these reducing equivalents in the electron transport chain then produces ATP. The complete oxidation of 1 mole palmitic acid thus results in the formation of 131 moles ATP. On a molar basis, this is 3.6 times more ATP generated (on a weight basis, 2.5 times more) compared to an equivalent amount of glucose.

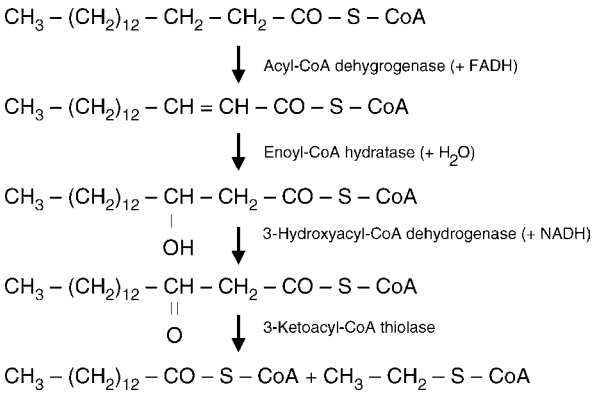



**FIGURE 5** Reactions of mitochondrial β-oxidation of saturated long-chain acyl-CoAs.

The first of the four reactions in mitochondrial β-oxidation of activated long-chain fatty acids is catalyzed by the acyl-CoA dehydrogenases, a group of enzymes with differing but somewhat overlapping chain-length specificity (very-long-chain, long-chain, medium-chain, and short-chain), resulting in the formation of 2-*trans*-enoyl-CoA. They are composed of four identical subunits, each of which contains a non-covalently bound flavin adenine dinucleotide (FAD). The enzyme is reoxidized via the electron-transferring flavoprotein and then in the electron transport chain by electron-transferring flavoprotein:ubiquinone oxidoreductase.

The second step of β-oxidation is catalyzed by 2-enoyl-CoA hydratases converting 2-*trans*-enoyl-CoA to 3-hydroxyacyl-CoA. This reaction is catalyzed by the membrane-bound long-chain trifunctional protein TFP, formerly known as the long-chain enoyl-CoA hydratase. The TFP is composed of two nonidentical subunits, the α and β subunits, present in a 1:1 molar ratio. The enzyme comprises the catalytic activities of 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase (α subunit) and 3-ketoacyl-CoA thiolase (β subunit). The soluble short-chain specific form, also known as crotonase, catalyzes the hydration of short-chain 2-enoyl-CoAs.

The third reaction is catalyzed by 3-hydroxyacyl-CoA dehydrogenases. At least two 3-hydroxyacyl-CoA dehydrogenase activities are found in the mitochondria. In addition to the TFP, there also is a short-chain specific form. The latter is composed of two identical subunits. Both activities require NAD as a cofactor. The NADH formed is then reoxidized by complex I of the electron transport chain.

The last reaction of the β-oxidation spiral is the thiolytic cleavage of 3-ketoacyl-CoA catalyzed by 3-ketoacyl-CoA thiolases. The general acyl-CoA thiolase and the long-chain 3-ketoacyl thiolase of the TFP have overlapping substrate specificities. The reaction is greatly in favor of product formation. There also is a third thiolase enzyme present in mitochondria called acetyl-CoA acetyltransferase or acetoacetyl-CoA thiolase. This enzyme is specific for acetoacetyl-CoA and 2-methylacetoacetyl-CoA and probably is involved in branched-chain amino acid metabolism, in ketogenesis, and in ketone body utilization.

The oxidation of (poly)unsaturated fatty acids requires auxiliary enzymes for their complete β-oxidation. Most unsaturated fatty acids have their double bonds in *cis* configuration with the first double bond between carbon atoms 9 and 10. Additional double bonds, if any, occur at three-carbon intervals.

Several cycles of β-oxidation yield a chain-shortened acyl-CoA with a *cis* double bond in 3, 4 position that is not a substrate for enoyl-CoA hydratase. Isomerization catalyzed by 2,3-enoyl-CoA isomerase leads to 2-*trans*-enoyl-CoA, which can undergo further β-oxidation, yielding after two additional cycles *trans*-2, *cis*-4-dienoyl-CoA, which also is a poor substrate for enoyl-CoA hydratase. The *cis*-4 double bond is reduced by the enzyme 2,4-dienoyl-CoA reductase, producing *trans*-3-enoyl-CoA, which is isomerized to *trans*-2-enoyl-CoA by 2,3-enoyl-CoA isomerase.

The rate of β-oxidation is adjusted to physiological needs and is subject to control in which substrate supply is important. The reaction catalyzed by CPT-I committing activated fatty acids toward oxidation in the mitochondria often is considered rate limiting. The regulatory property that imparts such control to this otherwise near-equilibrium reaction is the inhibition of CPT-I by malonyl-CoA. The muscle isoform of CPT-I is extremely sensitive to inhibition by malonyl-CoA, and it is thought that in heart and skeletal muscle, CPT-I is regulated only by changes in tissue malonyl-CoA concentrations. The liver isoform of CPT-I is much less sensitive to malonyl-CoA inhibition, and in liver the entry of activated fatty acids into the mitochondria is controlled by changes in the enzyme's sensitivity to malonyl-CoA as well as by changes in hepatic malonyl-CoA concentrations.

Although the mitochondrial uptake of long-chain activated fatty acids is absolutely carnitine dependent, the efficiency of the system is such that under physiological conditions, tissue carnitine concentrations seemingly have no significant impact on this process.

The control of β-oxidation flux by the redox state of NAD/NADH and FAD/FADH (ETF/$ETFH_2$) as well as by the acetyl-CoA/CoA ratio is important under some conditions.

## Regulation of Mitochondrial Acyl-CoA/CoA Ratio

β-oxidation is not the only pathway in which carnitine plays a role as a carrier of activated acyl groups. In conjunction with carnitine acetyltransferase and carnitine:acylcarnitine translocase, carnitine forms an effective shuttle system for acetyl groups out of the mitochondria, thus preventing sequestration of intramitochondrial free CoA. This acetyl-CoA buffer function allows CoA-dependent reactions such as pyruvate oxidation to proceed unimpaired. The CoA buffer function is not restricted to acetyl-CoA. Under normal physiological conditions, long-chain fatty acid movement is directed into the mitochondrial matrix. However, the carnitine:acylcarnitine translocase and CPT-II/carnitine acetyltransferase system can under certain conditions operate in the reverse direction, e.g., removing activated short-, medium-, and long-chain acyl groups from the matrix. This buffering has been demonstrated with isolated mitochondria as well as with cultured fibroblasts and lymphocytes from patients with defects in mitochondrial fatty acid oxidation. The accumulation of cellular acyl-CoAs as a result of an inborn error of fatty acid metabolism leads to a corresponding increase in acylcarnitines in the cell. In contrast to the acyl-CoAs, the acylcarnitines can be transported across the plasma membrane into the blood. The identification of these acylcarnitines in blood thus is a signpost for the metabolic defect.

## See Also the Following Articles

Coenzyme A • Fatty Acid Oxidation • Fatty Acid Receptors • Fatty Acid Synthesis and its Regulation

## Glossary

**carnitine** A water-soluble, small molecular weight compound derived from the essential amino acids lysine and methionine; involved in reversible transesterification reactions with acyl-CoAs of different chain length. It serves as a general transport vehicle for activated fatty acids.

**coenzyme A (CoA)** A cosubstrate of all acylcarnitine transferases catalyzing the reversible transesterification as well as of numerous other enzymes central to energy metabolism.

**mitochondria** Eukaryotic organelles surrounded by an inner and an outer membrane; they are the sites of energy production.

**β-oxidation** A series of enzyme reactions within the mitochondria, called the mitochondrial matrix, in which fatty acid chains are progressively shortened by the removal of two-carbon units as acetyl-CoA.

## Further Reading

Eaton, S. (2002). Control of mitochondrial β-oxidation flux. *Prog. Lipid Res.* **41**, 197–239.

Fraenkel, G., and Friedman, S. (1957). Carnitine. In *Vitamins and Hormones* (Harris, R. S., Marian, G. F. and Thiman, K. V., eds.) Vol. 15, pp. 73–118. Academic Press, New York.

Guzman, M., and Geelen, M. J. H. (1993). Regulation of fatty acid oxidation in mammalian liver. *Biochim. Biophys. Acta* **1167**, 227–241.

Kerner, J., and Hoppel, C. (1998). Genetic disorders of carnitine metabolism and their nutritional management. *Annu. Rev. Nutr.* **18**, 179–206.

Kerner, J., and Hoppel, C. (2000). Fatty acid import into mitochondria. *Biochim. Biophys. Acta* **1486**, 1–17.

McGarry, J. D., and Foster, D. W. (1980). Regulation of hepatic fatty acid oxidation and ketone production. *Ann. Rev. Biochem.* **49**, 395–420.

McGarry, J. D., and Brown, N. F. (1997). The mitochondrial carnitine palmitoyltransferase system. *Eur. J. Biochem.* **244**, 1–14.

Vaz, F. M., and Wanders, R. J. A. (2002). Carnitine biosynthesis in mammals. *Biochem. J.* **361**, 417–429.

Zammit, V. A. (1999). Carnitine acyltransferases: Functional significance of subcellular distribution and membrane topology. *Prog. Lipid Res.* **38**, 199–224.

## Biography

Charles L. Hoppel graduated from Gonzaga University and St. Louis University School of Medicine. He is Professor of Pharmacology, Medicine and Oncology at Case Western Reserve University, Co-Director of the Center for Inherited Disorders of Energy Metabolism at CWRU, and Associate Director, Research in the Geriatric Research, Education, and Clinical Center at the Louis Stokes VA Medical Center, Cleveland. His research interests are in mitochondrial structure and function, mitochondrial fatty acid oxidation, and regulation of carnitine palmitoyltransferase-I.

Janos Kerner graduated from Humboldt University in Berlin and held a faculty position in the Department of Biochemistry at Medical University of Pecs (Hungary) until 1994. He was a visiting faculty member in the Department of Biochemistry at Michigan State University and joined the faculty in the Department of Nutrition at Case Western Reserve University in 1995. His research interest is in mitochondrial fatty acid oxidation and its regulation.

# Caspases and Cell Death

**Don W. Nicholson**
*Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada*

**Pierluigi Nicotera**
*MRC Toxicology Unit, Leicester University, Leicester, UK*

**Gerry Melino**
*MRC Toxicology Unit, Leicester University, Leicester, UK*

**Caspases are cysteine proteases involved in cell death. The name "caspase" is derived from *c*ysteinyl aspartate-specific protease, i.e., proteases which cleave after an aspartate. The caspase family is comprised of 13 proteins in humans, seven in *D. melanogaster*, and a single protein in *C. elegans*. All caspases consist of three structural domains: a prodomain, a large subunit, and a small subunit. The prodomain is important for the regulation of the enzymatic activation of caspases; the active enzyme consists of the small and large subunits together. To obtain an enzymatically active enzyme, the three subunits must be proteolytically cleaved, which is effected by other endoproteases, normally also a caspase. This article discusses caspases in the context of cell death.**

## History and Classification

Caspases were first identified in 1989 by Sleath and Schmidt as a protease required for the activation of interleukin-1$\beta$. In 1993, the group of Bob Horvitz discovered that the cell death gene ced-3 is a protease belonging to the same family. In 1999, the first human genetic disease caused by mutation of a caspase (type 10) was identified by Michael Lenardo.

Phylogenetic analyses, and also studies of substrate specificity, indicate that caspases can be subdivided into three different groups. Figure 1 reports the present classification of the human caspases.

Group 1 includes caspases 1, 4, 5, and 12, which are involved in inflammation. The prototype of this group is caspase 1, previously known as interleukin conversion enzyme 1$\beta$, or ICE. Its enzymatic activity was identified in 1989, but the enzyme was only purified and cloned in 1992, the same year in which Lois Miller identified the first inhibitor, the viral protein CrmA. Caspase 1, or ICE, controls the maturation of IL-1$\beta$, actively participating in the inflammatory response. Site P4 of the substrate is preferentially a hydrophobic residue.

Group 2 includes caspases 2, 3, and 7, shown by a star in Figure 1. The prototype of this group is caspase 3, the homologue of ced-3 in *C. elegans*. Group 2 caspases are directly involved in apoptosis in the terminal effector phases. Group 2 caspases have a very small prodomain, indicating a simple regulation of their enzymatic activation. These caspases are in general activated directly by another caspase, in a cascade of enzymatic amplification. Site P4 of the substrate is preferentially an Asp (D) residue. Site P3 of the substrate is preferentially a Glu (E) residue. Therefore, this subfamily preferentially cleaves substrates with the sequence DExD.

Group 3 consists of caspases 6, 8, 9, and 10. These caspases have a very large prodomain, and therefore a complex mechanism of activation. In fact, these caspases need molecular adaptors to be enzymatically activated, in contrast to group 2 caspases. Caspases 8 and 10 are part of the signal transduction mechanism of apoptosis receptors such as CD95; in this case, the adaptor molecule is FADD. Caspase 9 is activated in the apoptosome, and the necessary adaptor molecule is Apaf-1. Site P4 of the substrate is preferentially an aliphatic residue.

## Structure and Active Site

As already mentioned, caspases consist of three structural domains: a prodomain, a large subunit (also named as p20), and a small subunit (called p10).

The enzyme is constituted of the small and large subunits (p20/p10, ~30 kDa). In fact, the active site is mostly in the p20 subunit: it can be further classified into four different subsites, S4 to S1. The S1 subsite, which attacks the carboxyl group of the polypeptide side chain in P1 (the site of cleavage is Asp-D), is composed of p20 as well as p10 subunits. Similarly, the site for recognition and positioning of the substrate (S4–S1) is furnished by both p20 and p10, even though the residues mostly

 © 2004, Elsevier Inc. All Rights Reserved. 

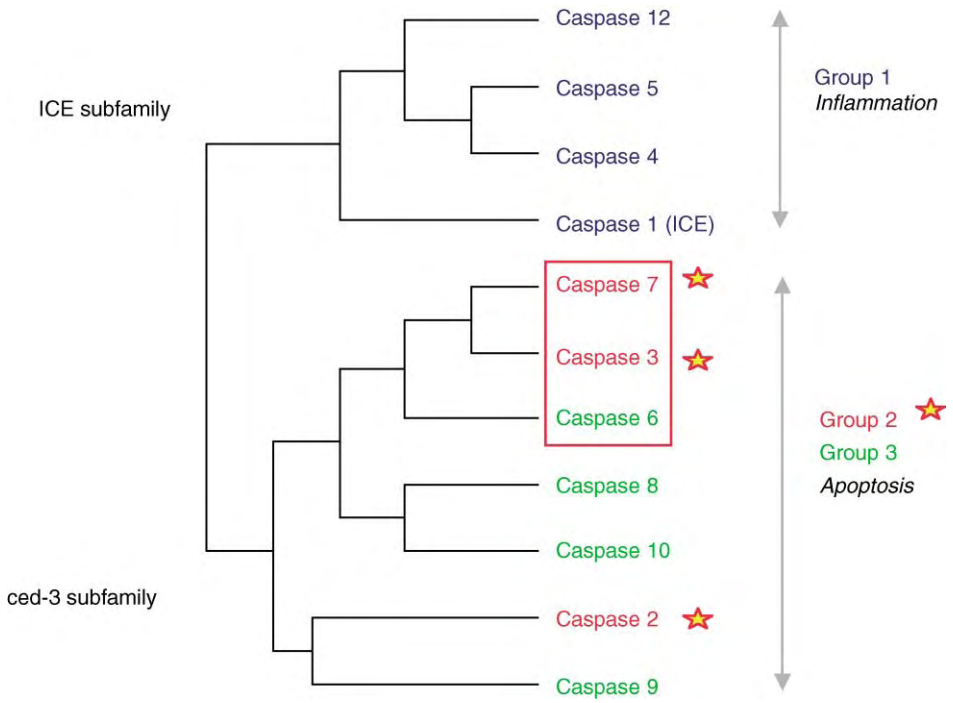



**FIGURE 1** The human caspases family. Classification of the human caspases. According to the phylogenic tree, caspases are subdivided in two major subfamilies, ICE and ced-3. The former are involved in inflammation, and the latter in apoptosis. The caspases with a very short prodomains (<30 a.a.) are boxed (types 3, 6, 7); all the other enzymes have long prodromains (>10 kDa) subject to complex regulation. The enzymes can be divided according to their proteolytic specificity into group 1, involved in cytokine maturation, group 2, involved in the final effector phase of apoptosis (indicated by a star), and group 3, involved in the upstream regulation of apoptosis. At least two gene clusters have been identified, consistent with some caspases arising from tandem gene duplication. Caspase 13 is an error of sequencing; caspase 12 is mutated into a nonfunctional enzyme in up to 90% of the human population; caspase 11 is involved in neuronal death; caspase 14 is involved in skin differentiation. Please note that caspases are not indicated in the scheme. Modified from Nicholson, D. W. (2000). From bench to clinic with apoptosis-based therapeutic agents. *Nature* **407**, 810–816.

responsible for specificity (S4) are contained in p10 (Figure 2).

Caspases enzymatically function as dimers of two identical molecules bound to each other. Figure 2 shows a very schematic view of the dimer formation.

Each monomer (p20/p10) is formed by a compact cylinder dominated by six $\beta$-sheets and five $\alpha$-helices distributed on the opposite site of the plane formed by the $\beta$-sheets. The dimer (p20/p10)2 contains two (p20/p10) units aligned head to tail, which therefore position their respective active sites on opposite sides of the molecule. While there are two active sites, their cooperation has never been observed. The orientation of the molecule indicates a specific mechanism of activation, well regulated with security systems (a "safety catch").

## Substrate Recognition

The substrate recognition site of the caspase is indicated as S4–S1, at which the substrate is bound at residues P4–P1 (Figure 3). This recognition guarantees an absolute specificity. The S4–S1 substrate recognition site varies in each caspase, even if S1 is absolutely constant and highly tight. This is responsible for the absolute specificity to bind Asp (D) as the substrate residue P4. In fact, Asp is physically constrained and held by a hydrogen bond with Arg179, Gln283, and Arg341 (the numbers refer to caspase 3). The dimensions of the S1 subsite are reduced to a minimum and are therefore responsible for the lack of tolerance for P1 (Arg). Bond sites P2 and P3 (subsites S2 and S3) are more tolerant, less specific, and more heterogeneous in the various caspases. Therefore, the substrate (or the inhibitor) positions itself within the S1–S4 site, creating interactions as hydrogen bonds with Ser339 and Arg341 (conserved in practically all of the caspases). The P4 (S4) binding site is the major determinant of specificity.

## Mechanism of Action

Caspases are cysteine proteases and, therefore, possess a catalytic triad, which includes a cysteine (active site) in the vicinity of a histidine. The third component of the classical catalytic triad typical of cysteine proteases is

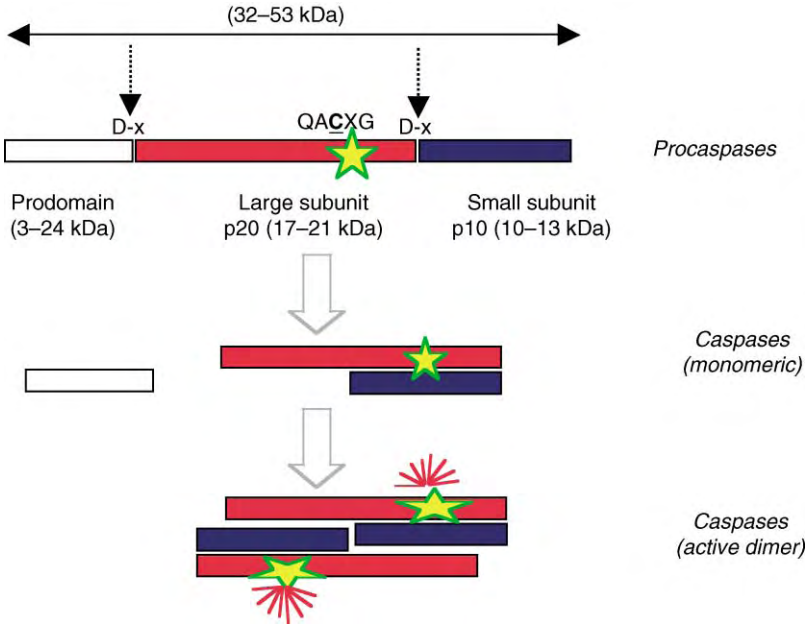



**FIGURE 2** Structural organization and activation of caspases. The caspases are produced as proenzymes (32–53 kDa), including a prodomain (3–24 kDa), the large subunit (17–21 kDa) containing the active site, and the small subunit (10–13 kDa). To become enzymatically active, these three components must be proteolytically cleaved (D-x site), a phenomemon that is regulated by the prodomain itself; this allows the assembly of the large and small subunits, with the dimerization into a functional enzyme.

not constant in caspases, and its function can be substituted by different groups in different components of the family. For example, if we consider caspase 3, subsequent to substrate binding, the catalysis can take place with the catalytic diad Cys285 and His237. In fact, the third element of the cysteine protease triad is substituted by an ossianionic bond formed with Gly238 and Cys285 (conserved in all caspases).

## Sequence of Action

While the regulatory caspases of the death receptor (types 8 and 10) or of the apoptosome (type 9) participate in signal transmission, the effector caspases (types 3, 6 and 7) determine the death of the cell. Effector caspases are diverse, and are not activated in parallel, but in a sequential manner, resulting in an amplification cascade of proteolytic cleavage (vaguely similar to the cascade of proteolytic activation and amplification of the complement system, or blood coagulation). These cascades have the effect of augmenting the signal.

In the apoptosome, the first caspase to be activated is caspase 9. This in turn activates caspases 3 and 7. Caspase 3 is also able to proteolytically activate caspase 9, therefore creating a reciprocating loop of potentiation.

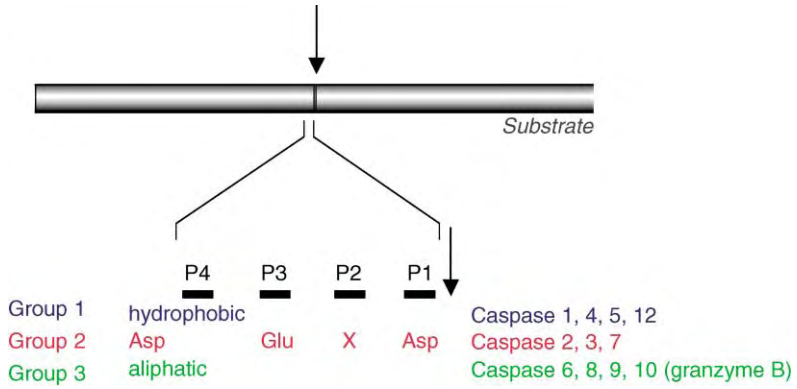



**FIGURE 3** Caspase proteolytic specificity. The caspases recognize a tetrapeptide motif corresponding to the four residues P4–P3–P2–P1. While the positions at P3 and P1 seem to be obligatory, the position P4 allows the classification of three subfamilies (Figure 5). This property has facilitated the identification of group-specific inhibitors.

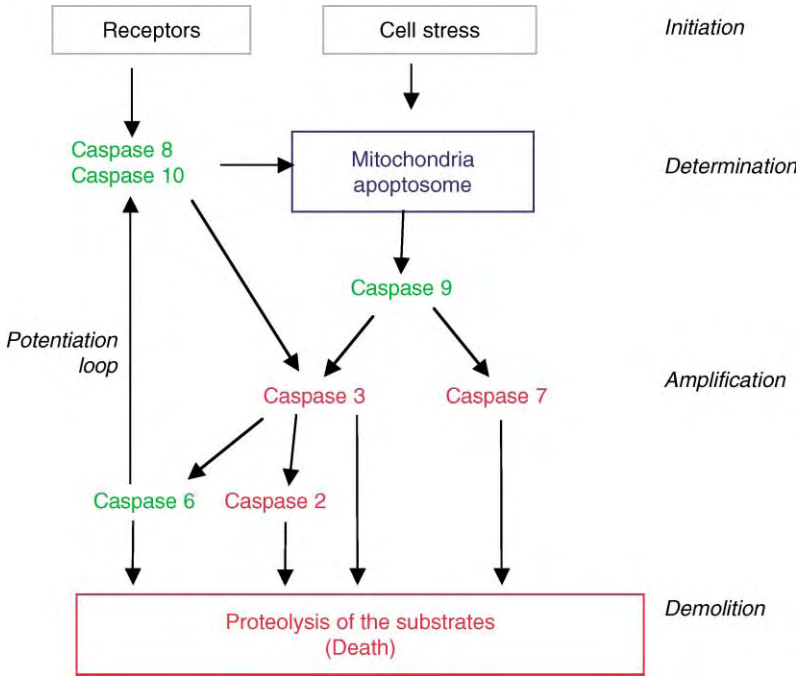



**FIGURE 4** Sequential activation of caspases. In general, caspases with a long prodomain are involved in the upstream regulation and activation of the apoptotic pathway; they require a tight regulation and activation; furthermore, they cleave very specific substrates. Caspases with a short prodomain are involved in the amplification of the effector cascade, thus they have a very simple, fast, and direct activation, and they also have many substrates, inactivation of which finally kills the cell. Caspase 6 seems to be able to reactivate the upstream regulatory caspases, creating a feedback forward potentiation loop that strongly enhances the amplification of the death signal. Color code is in keeping with Figure 4. Modified from Slee, E. A., Adrian, C., and Martin, S. J. (1999). Serial killers: Ordering caspase activation events in apoptosis. *Cell Death Diff.* **6**, 1067–1074.

Caspase 3 proteolytically activates caspases 6 and 2. At this point, caspases 3, 7, 6, and 2 are highly activated and work to proteolytically cleave their intracellular substrates and determine cell death itself. A diagram of this order of activation is depicted in Figure 4.

Caspase 6 can also proteolytically activate the apical regulatory caspases 8 and 10, therefore creating a further loop of potentiation, which further enhances the death receptor signals. Figures 5–7 show the caspase activation, the apoptic pathway in humans, and the regulation of the apical regulatory caspases.

Schematically we can redefine the molecular phases of apoptosis in the following manner: (1) "initiation," activation of death receptors (caspases 8 and 10), (2) "determination," activation of the apoptosome (caspase 9), (3) "amplification," increase in the type and number of active caspases (caspases 3, 7, 6, and 2) and apical reactivation loop (caspases 8 and 10), and (4) "demolition," proteolytic cleavage of over 250 different substrates which determine the death of the cell (Figure 4).

## Substrates during Apoptosis

Experimental studies indicate that over 700 different polypeptides can be generated during apoptosis. Up to

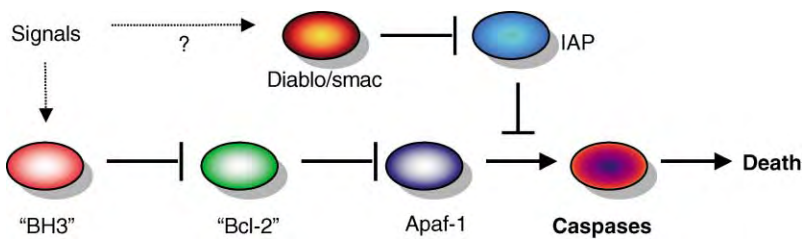



**FIGURE 5** Caspases and cell death. Caspases are the proteolytic enzymes responsible for cell death. They are activated by an adaptor molecule (apaf-1), which in turn are controlled by the Bcl-2 family, and in particular by the "BH3-only" subfamily and the Bcl2-subfamily. The final stages of activation of the caspase can be regulated by a different pathway, the IAPs. This simple scheme is conserved in evolution from *C. elegans* and *D. melanogaster.*

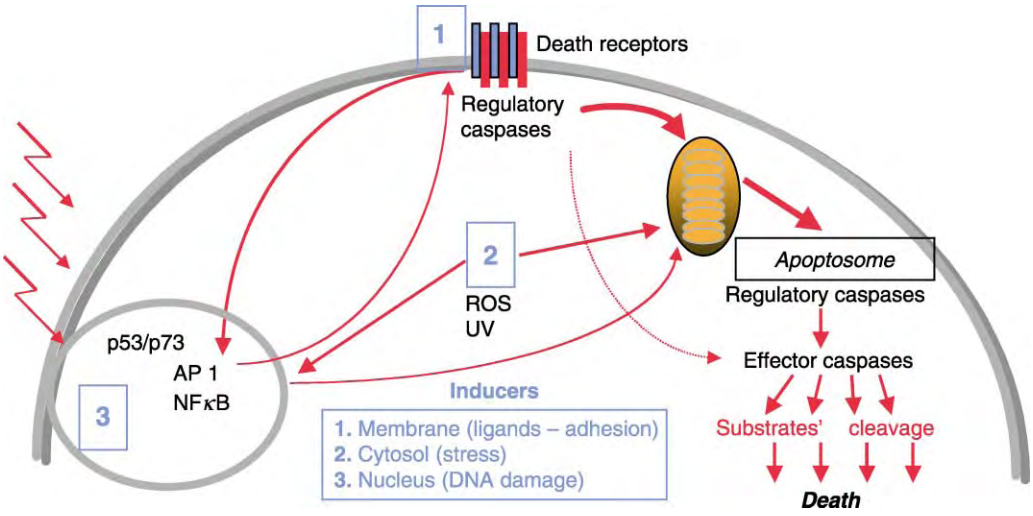



**FIGURE 6** The apoptotic pathways in humans. In mammalian cells, apoptosis can de triggered at three different levels, as indicated. The signal converges to the apoptosome were the final effector phase occurs. Caspases are involved both in the upstream regulatory phase, and in the final terminal phase. The regulation at the level of the death receptor, and of the apoptosome is shown in greater detail in Figure 7.

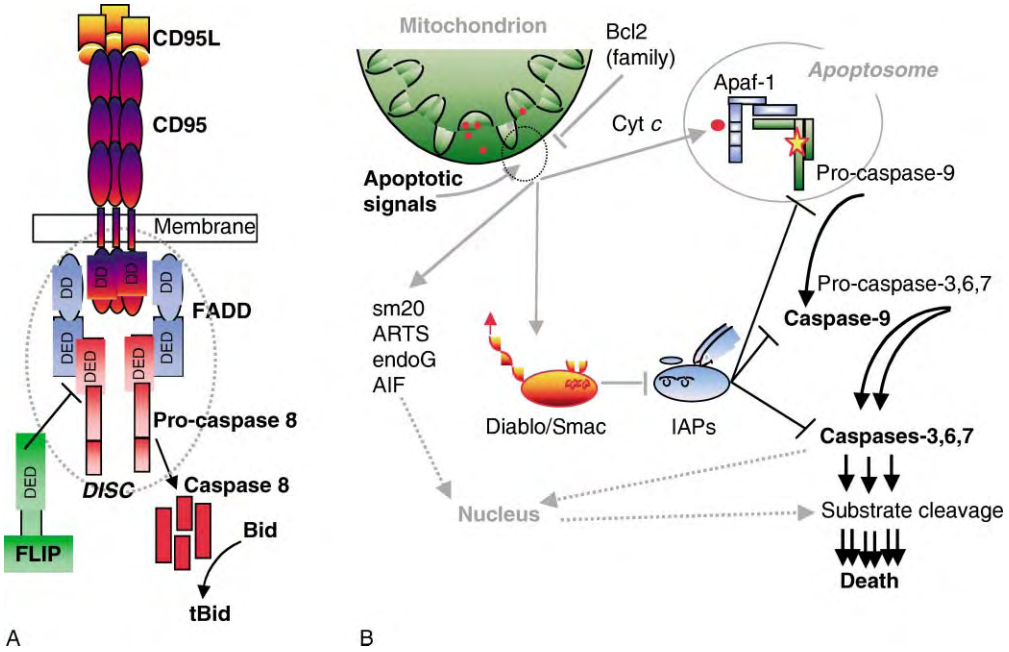



**FIGURE 7** Regulation of the apical regulatory caspases. The general scheme is reported as Figure 6. (A) Formation of the death inducing signaling complex (DISC), following the activation of a death receptor such as CD95 (also called Fas or APO-1). Three molecules of ligand (CD95L) bind three molecules of receptor (CD95), allowing the recruitment of the adaptor molecule FADD via their death domain (DD). In turn FADD, via its death effector domain (DED), recruits and activates caspase 8, which cleaves the specific substrate Bid. Truncated Bid (tBid) is in fact the molecular signal that propagates the death signal to the mitochondria and the apoptosome. This mechanism can be inhibited by the molecule FLIP, via its DED. (B) When activated by apoptotic signals (e.g., by tBid), mitochondria release several molecules, as indicated. In particular, cytochrome *c* goes to the apoptosome (formed by cytochrome *c*, Apaf-1, pro-caspase 9, and dATP), and allows the activation of caspase 9. This in turns activates several molecules of downstream effector caspases (types 3, 6, 7), and consequently the cleavage of many cellular substrates results in cell death. Still at its terminal stage, cell death can be inhibited by specific inhibitory proteins (IAPs), but another mitochondria-released protein, Diablo/Smac, can remove the protection by IAPs. The cascade of proteolytic amplification created by caspase 9 (apical regulatory caspase) and caspases 3, 6, and 7 (downstream effector caspases) is extremely powerful.

2003, according to Schultze Ostoff, over 250 proteins have been found to be cleaved by caspases during the execution of apoptosis.

Table I indicates some of the principal substrates. The proteolytic cleavage of these substrates clearly renders the cell incapable of performing its proper functions.

## Inhibitors

For practical reasons we distinguish pharmacological (Table II) and natural (Table III) inhibitors of caspases.

**TABLE I**

**Caspase Substrates**

| *DxxD site* | *non-DxxD Site* |
|---|---|
| DNA-PKs | STAT1 |
| PARP | Sp-1 |
| Rad 51 | SRP p72 |
| Acinus | NFκB |
| CAD/DFF45 | PITSLRE kinases |
| DNA-RTC140 | PAK-2 |
| Rb | p59 fyn |
| mdm2/HDM | CaMK-IV |
| p21-waf1/cip | p28 Bap31 |
| NuMA | Actin |
| ATM | Gas2 |
| U1-70KsnRNP | Lamin A and B |
| hnRNP-C1/C2 | Bcl-XI |
| SREB | Bid |
| IκB-alfa | APP-beta |
| D4-GDI | Pro-IL-16 |
| cPLA2 | Pro-caspases |
| PKC-delta, -theta, -zeta | |
| MEKK-1 | |
| Mst1 | |
| PRK2 | *Unknown site* |
| PP2A | wee1 |
| FAK | CDC27 |
| Fodrine (all) | SAF-A/hnRNP-U |
| Gelsolin | hnRNP-A1 |
| Keratin-18 | Ras GAP |
| LAP2 | Raf 1 |
| Nup153 | Akt 1 |
| Rabaptin-5 | Cbl e Cbl-b |
| APC | PKN |
| Hsp90 | Catenin-beta, -gamma |
| UbqCE NEDD4 | Kinectin |
| Bcl 2 | Caplastatin |
| Presenilin 2 | Ataxin-3 |
| Huntigtin | AMPA receptors |
| SBMA-AR | p27 kip1 |
| Atrofin-1 | MCM3 |

The substrates of caspases can have single cleavage site (e.g., DQTD in Gelsonin), nested multiple sties (e.g., DEVDGVD in PARP), redundant clustered sites (e.g., DSLD-(X13)-DEED-(X16)-DLND-(X32)-DGTD in Huntigtin), or distal multiple sites (e.g., DEPD and DAVD in ICAD).

**TABLE II**

**Peptide Inhibitors of Caspases**

| | zVAD-fmk | aDEVD-cho | aYVAD-cho |
|---|---|---|---|
| Caspase 1 | 2 | 15 | 1 |
| Caspase 2 | 2400 | 1700 | >10 000 |
| Caspase 3 | 40 | 1 | >10 000 |
| Caspase 4 | 130 | 130 | 360 |
| Caspase 5 | 5 | 200 | 160 |
| Caspase 6 | 100 | 30 | >10 000 |
| Caspase 7 | 40 | 2 | >10 000 |
| Caspase 8 | 2 | 1 | 350 |
| Caspase 9 | 4 | 60 | 1000 |
| Caspase 10 | | 10 | 400 |

Inhibition constant (Ki, nM) shown is t1/2 at 1 μM in seconds.

Modified from Ekert, P. G., Silke, J., and Vaux, D. L. (1999). Caspase inhibitors. *Cell Death Diff.* **6**, 1081–1086.

The understanding of the molecular mechanism of catalysis and of the properties of the sites P4–P1 of the substrate were the basis for designating pharmacological inhibitors, both of polypeptide type and of low molecular weight compounds. The electrophylic groups, which interact irreversibly with the catalytic Cys are aldehydes, nitriles, and ketones, of which the latter are much more stable than the others *in vivo* and therefore are most easily applied to pharmaceutics. Caspase inhibitors are all constituted according to the following schema: [tetrapeptide]-C-CH2-X. Here X signifies a –Cl or –F (fluoro- or chloro-methyl ketone, fmk), -N2 (diazomethylketone), or –OCOR (acyloxymethylketone). The peptide portion (stabilized), determines the specific selectivity of the various caspases. Thus, zVAD-fmk is a nonspecific inhibitor of almost all of the caspases. DEVD-fmk, on the other hand, is specifically selective for effector caspases such as caspases 3, 6, and 7. Table II gives an idea of the specific inhibitors for different caspases.

In 1991, Lois Miller first identified a natural inhibitor of apoptosis, before even the discovery of caspases. The protein p35 produced by baculovirus (*Autographa californica*) is a potent and selective inhibitor of caspases. Subsequently, numerous other viral caspase inhibitors were identified, such as cytokine response modifier A (CrmA). Inhibition of caspases by these specific proteins is one of the important pathways among which viruses regulate apoptosis.

Numerous cellular proteins exist with actions similar to those of these viral caspase inhibitors. They are called IAPs (inhibitors of apoptosis). Table III shows the different viral and human IAPs with their relative inhibitory effects on each caspase.

All of the proteins which inhibit caspases, whether viral or cellular, are characterized by structural domains

**TABLE III**

**Natural Caspase Inhibitors**

| | Viral | | | Cellular | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | crmA | p35 | OpIAP | XIAP | cIAP1 | cIAP2 | Survivin | NAIPPI9 | GBI |
| Caspase 1 | 0.01 | 9 | Inib | NI | NI | NI | | | NI |
| Caspase 2 | >10 000 | Inib | Inib | | | | | | |
| Caspase 3 | 1 600 | 0.1 | NI | 0.7 | 100 | 35 | Inib | NI | |
| Caspase 4 | 1 | | | | | | | | |
| Caspase 5 | 0.1 | | | | | | | | |
| Caspase 6 | 110 | 0.4 | | NI | NI | NI | | | |
| Caspase 7 | >10 000 | 2 | | 0.2 | 40 | 30 | Inib | NI | |
| Caspase 8 | 0.8 | 0.5 | | NI | NI | NI | NI | | NI |
| Caspase 9 | 2 | | | Inib | Inib | Inib | | | |
| Caspase 10 | 17 | 7 | | | | | | | |
| Granzyme B | NI | NI | | | | | | | Inib |

Inhibition constant (Ki, nM) shown is t1/2 at 1 μM in seconds. NI = noninhibitory, Inib = inhibitory.

Modified from Ekert, P. G., Silke, J., and Vaux, D. L. (1999). Caspase inhibitors. *Cell Death Diff.* **6**, 1081–1086.

called BIR (baculovirus IAP repeat), consequently all IAP proteins are also called BIR proteins (BIRp). On the other hand, the contrary does not hold: there are BIR proteins which do not show IAP activity. This fact is due to the prokaryotic origin of BIR proteins, which are multifunctional. Many BIR proteins in humans (e.g., survivin) are passenger proteins in the mitotic spindle and are implicated in cell division.

## Caspase Knockouts

The creation of transgenic mice lacking specific caspases has been accomplished for most caspases. Table IV displays the phenotypes for these mice. Because caspases play a determining role in apoptosis, sometimes specifically, sometimes redundantly, the phenotypes are extremely diverse, as indicated by the table.

The role of caspases in the receptor mechanism is indicated by the caspase 8 knockout.

The similarity of the phenotypes of the knockouts of caspases 3, 9, and Apaf-1 indicate the presence of a very important mechanism in the apoptosome by which the amplification of the caspase cascade is initiated and regulated.

**TABLE IV**

**Caspase Knockouts**

| | Development | Apoptotic phenotype |
|---|---|---|
| Caspase 1 | Normal | Inflammation (CD95?) |
| Caspase 2 | Normal | Germinal cells (stem cells?) |
| Caspase 3 | Lethal PN | Neural |
| Caspase 6 | Normal | |
| Caspase 7 | Lethal E | |
| Caspase 8 | Lethal E | Death receptors (CD95, TNF, DR3) |
| Caspase 9 | Lethal E | Neural |
| Caspase 11 | Normal | Thymocytes, neural (CD95?) |

Often, the severity of the phenotype depends on the genetic background of the animals, as, for example, described in caspase 3.

E = embrionically lethal; PN = perinatally lethal.

## Pathological Implications

The execution of apoptosis is dependent on the activation of caspases, and as a result, caspases play a determining role in all pathologies of excessive apoptosis. For example, caspases play a crucial role in the *in vivo* activation of death described in myocardial infarction and in cerebral ischemia, conditions in which cell death occurs both by apoptosis as well as by necrosis.

Caspases can also play an aberrant role in regulatory processes determining the major propensity or vulnerability of cells to lethal insults. Examples are the possible role played by caspases in triplet diseases such as Huntington's and in neurodegeneration like in Alzheimer's disease.

In the case of Huntington's disease, low level of intracellular activation of caspase 3 liberates an amino terminal fragment of the protein Huntington (four DxxD sites in a cluster), containing the Glu expansion, facilitating a pathological aggregation. In turn, this

aggregate sensitizes more caspase 3, forming a vicious cycle of activation and aggregation of poly-Glu. The cell, thus sensitized, facilitates the activation of caspase 8 (from the CD95 receptor, or directly by an effector caspase, or even stimulated indirectly by the poly-Glu aggregate), determining the death of the cell. This sensitization can also occur extremely slowly (years).

In Alzheimer's disease, caspase 3 alters the normal processing of the amyloid $\beta$-precursor protein (APP), removing the carboxy terminal. APP, deprived of the intramolecular reinternalization signal, proceeds down a route of degradation that results in the formation of the amyloid beta (A$\beta$) peptide, which can in turn favor the activation of apoptosis by means not yet clearly understood.

In either case, caspase 3 seems to augment the baseline levels (threshold?) of activation of cell death, rendering the cell more sensitive to death, and participating therefore in the pathogenetic mechanisms of these important illnesses.

## Therapeutic Outlook

Selective peptide inhibitors of different caspases (Table II) have been successfully developed, even though they show serious problems penetrating the cell membrane, as well as having electrophyllic promiscuity and stability in solution, which makes them susceptible to attacks by biological nucleophiles, such as catepsin. Because of this, they are not easily adaptable to clinical use; this has allowed scientists to obtain many interesting *in vitro* results. More recently, Merck (like Smith Kline Beecham, BASF, Idun/Novartis, and Vertex) has in advanced stages of development, new classes of low molecular weight nonpeptide inhibitors. These show a much higher potency and specificity, opening new therapeutic perspectives. Nonpeptide inhibitors like "isatins" attain potencies 100–1000 times greater than the class of zVAD-fmk, with substantial improvements *in vivo*. These studies are still in early stages, and we hope that the near future will tell as the best clinical application for this new class of drugs.

## See Also the Following Articles

Amyloid • Bax and Bcl2 Cell Death Enhancers and Inhibitors • Cell Death by Apoptosis and Necrosis

## Glossary

**apoptosis** The physiological mechanism of programmed cell death mediated by caspases (cysteine proteases), regulated by the Bcl-2 family of proteins, and triggered from the membrane (death receptors, adhesion), cytosolic stress, or nuclear signals (DNA damage). Excessive or defective apoptosis is involved in diseases such as ischemia, AIDS, neurodegeneration, autoimmunity, and cancer.

**apoptosome** Multiprotein complex of 700 kDa molecular weight formed by procaspase-9, apaf-1, dATP, and cytochrome *c*. It proteolytically activates caspase-9, leading to the activation of downstream-effector caspases, and subsequently death of the cell.

**BIR** Baculovirus IAP Repeat. A protein-structural domain, conserved in both mammalian and viral proteins, able to react with the apoptosome or with the active caspases. It characterizes the IAP proteins.

**caspase** Cysteinyl *asp*artate-specific prote*ase*, cysteine proteases which cleave after an aspartate (D). The family includes 13 enzymes involved either in programmed cell death (apoptosis) or in inflammation.

**death receptors** Cell membrane receptors (e.g., CD95, TRAIL-R, TNF) belonging to the TNF (tumor necrosis factor) superfamily, able to activate apoptosis via the formation of a DISC when bound by their specific ligands (e.g., CD95L, TRAIL, TNF). Essential in regulating immune responses.

**DISC** Death initiation signaling complex. Multiprotein complex beneath the cell membrane activated by trimeric death receptors (e.g., CD95, TRAIL-R, TNF). It includes the receptor, adaptor molecules, procaspases (caspases 8 and 10), and their related substrates (e.g., Bid) that signal to the mitochondria-apoptosome to activate the caspases, and therefore kill the cell by apoptosis.

**IAP** *I*nhibitors of *ap*optosis. Mammalian or viral proteins able to inhibit the activation of the apoptosome or the active caspases. They are characterized by structural domains called BIR; consequently all IAP proteins are also called BIR proteins (BIRp).

## Further Reading


Black, R. A., Kronheim, S. R., and Sleath, P. R. (1989). Activation of interleukin 1$\beta$ by a co-induced protease. *FEBS Lett.* **247**, 386–390.

Ekert, P. G., Silke, J., and Vaux, D. L. (1999). Caspase inhibitors. *Cell Death Diff.* **6**, 1081–1086.

Fischer, U., Laenicke, R. U., and Schultze-Ostoff, K. (2003). Many cuts to ruin: A comprehensive update of caspase substrates. *Cell Death Diff.* **10**, 76–100.

Kostura, M. J., Tocci, M. J., Limjuco, G., Chin, J., Cameron, P., Hillman, A. G., Chartrain, N. A., and Schmidt, J. A. (1989). Identification of a monocyte specific pre-interleukin 1$\beta$ convertase activity. *PNAS-USA* **86**, 5227–5231.

Melino, G. (2001). The Syren's song (Concept: Apoptosis). *Nature* **412**, 23.

Nicholson, D. W. (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Diff.* **6**, 1028–1042.

Nicholson, D. W. (2000). From bench to clinic with apoptosis-based therapeutic agents. *Nature* **407**, 810–816.

Slee, E. A., Adrian, C., and Martin, S. J. (1999). Serial killers: Ordering caspase activation events in apoptosis. *Cell Death Diff.* **6**, 1067–1074.

Wang, J., Zheng, L., Lobito, A., Chan, F. K.-M., Dale, J., Sneller, M., Yao, X., Puck, J. M., Strauss, S. E., and Lenardo, M. J. (1999). Inherited human caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell* **98**, 47–58.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993). The *C. elegans* cell death genes ced-3 encodes a protein similar to mammalian interleukin 1$\beta$-converting enzyme. *Cell* **75**, 641–652.

Zheng, T. S., Hunot, S., Kuida, K., and Flavell, R. A. (1999). Caspase knockouts: Matter of life and death. *Cell Death Diff.* **6**, 1043–1053.

## Biography

Gerry Melino is Editor in Chief of the journal *Cell Death Differentiation* (www.nature.com/cdd) as well as group leader at the Medical Research Council, Toxicology Unit in Leicester, UK, and Professor at the University of Rome "Tor Vergata." His research is mainly focused on the p53 family and in particular on p73 and p63.

Pierluigi Nicotera is the Director of the Medical Research Council, Toxicology Unit in Leicester, UK, and his research interest is on neurodegeneration and neurotoxicology.

Don W. Nicholson is Vice President of Merck, responsible for the San Diego (California, USA) research site dedicated to neurodegeneration. He is a major contributor to the enzymology of caspases, and to drug development of low molecular weight inhibitors of caspases.

# Cell Cycle Controls in $G_1$ and $G_0$

**Wenge Shi and Steven F. Dowdy**
*University of California, San Diego School of Medicine, La Jolla, California, USA*

**The cell cycle is the process by which one cell becomes two. Somatic cell division involves cell growth (increase in cellular components, such as ribosomes, membranes, organelles) throughout the cell cycle, faithful replication of its DNA during the S phase of the cell cycle, and precise distribution of DNA between daughter cells during the mitosis (M) phase. In addition, with the exception of early embryonic cell divisions, two gap phases, Gap 1 ($G_1$) and Gap 2 ($G_2$), are separated by S phase. As multicellular eukaryote organisms regulate cell division tightly to maintain tissue homeostasis, the most important regulatory decision is made during exiting of the resting state so called the $G_0$ phase and $G_1$ phase of the cell cycle before cells become committed to initiate DNA synthesis and complete the cell cycle.**

## $G_1$ and $G_0$ Phase of the Cell Cycle

The majority of cells in adult metazoans are permanently withdrawn from the cell cycle in a terminally differentiated state. Only small numbers of cells, such as hematopoietic and epithelial early progenitor cells, are actively proliferating. Other cells are reversibly withdrawn from the cell cycle and remain in a quiescent stage or the $G_0$ phase of the cell cycle. Upon proper stimulation, these cells can re-enter the cell cycle. For example, highly differentiated hepatocytes in adult liver are present in the $G_0$ resting phase and rarely replicate normally. However, in response to acute liver injury or distress, these $G_0$ hepatocytes can be stimulated to re-enter the cell cycle and regenerate the liver. Similarly, primary peripheral blood lymphocytes (T and B cells), which are present in the $G_0$ phase, can re-enter the cell cycle and start clonal expansion when presented with the appropriate antigen. These examples serve to demonstrate that certain cell types may enter and exit the cell cycle pending the appropriate stimulus, whereas the vast majority of cells in a matured metazoan have permanently exited the cell cycle.

The most important decision for cell cycle progression is made during the $G_1$ phase. In $G_0$ phase, cells respond to extracellular signals by entering the $G_1$ phase of the cell cycle. However, prior to transiting into the late $G_1$ phase, they must traverse the growth factor-dependent restriction point. This is a critical regulatory phase of the cell cycle where a cell becomes committed to enter S phase and finish the remaining cell cycle. In contrast to normal cells, tumor cells are often less growth factor-dependent and fail to respond to growth inhibitory signals. Consequently, tumor cells select for genetic mutations that disrupt the important decisions performed at the restriction point and this is, in fact, one of the hallmarks of cancer.

## Regulators of $G_0$ Exit and $G_1$–S Progression

One of the key negative regulators of the restriction point and early $G_1$ to $G_0$ cell cycle exit is the retinoblastoma tumor suppressor protein (pRb) and two closely related family members, p107 and p130. Murine embryonic fibroblasts (MEF) deficient for all three pocket proteins fail to respond to $G_1$ phase arrest signals following contact inhibition, serum starvation, or DNA damage. The antiproliferation function of the pocket proteins depends, at least in part, on their interaction with E2F/DP transcriptional factors, which regulate the expression of key genes required for DNA synthesis, DNA repair, DNA-damage checkpoint, apoptosis, and mitosis. Direct interactions of pRb family members with E2F/DP complexes and the recruitment of chromatin-modifying enzyme complexes, such as histone deacetylases (HDAC), polycomb group proteins, the SWI/SNF complex, and histone methyl transferases, to E2F-reponsive genes results in inhibition of target gene expression.

Distinct pRb family member–E2F repressor complexes exist in different cell cycle phases. As an example, during the $G_0$ phase, p130 and p107 interact with E2F4 and E2F5, whereas pRb remains unbound. E2F4 and E2F5 are expressed constitutively and are involved in E2F-dependent gene repression during cell cycle exit and terminal differentiation. It is also reported that E2F6, the sole E2F family protein that does not interact with the pocket proteins, mediates gene repression with polycomb group proteins in the $G_0$ phase. Further studies are

 © 2004, Elsevier Inc. All Rights Reserved. 

needed to resolve the contributions of E2F6 versus E2F4 and E2F5 for maintaining quiescence. In contrast, during early $G_1$ phase, pRb interacts with E2F1, E2F2, and E2F3, the so-called "activator E2Fs." When the cell passes through the restriction point, pRb becomes inactivated by hyperphosphorylation, releasing E2Fs 1–3 and promoting the activation of E2F-responsive genes for cellular proliferation. Not surprisingly, MEFs deficient for these E2F1, E2F2, and E2F3 exhibit impaired E2F-responsive gene induction in response to serum and significant proliferative defects.

pRb undergoes cell-cycle-specific phosphorylation through the $G_1$ and S phases of the cell cycle and subsequent dephosphorylation during mitosis. Hypophosphorylated (low level of phosphorylation) pRb is the active, transcriptional repressing form of pRb and can simultaneously bind to both E2F and HDAC. Hypophosphorylated pRb is also selectively bound and inactivated by DNA tumor virus oncogenes, SV40 large T antigen, adenovirus E1A protein, and human papillomavirus (HPV) E7 protein.

pRb is physiologically regulated by a group of serine/threonine-specific protein kinases called cyclin-dependent kinases (Cdks). Specifically, cyclin D:Cdk4/6 and cyclin E:Cdk2 kinases are responsible for phosphorylating pRb during the $G_1$ phase of the cell cycle. However, cyclin D and cyclin E are regulated differently and play distinct roles in the cell cycle regulation of pRb (Figure 1). D-type cyclins are not expressed in $G_0$ cells. However, cyclin D levels increase after stimulation of growth factors as a result of increased transcription mediated by Myc and Ras/MEK/MAPK signaling pathway and increased stability mediated by the PI(3)K/Akt signaling. In cycling cells, cyclin D levels and associated kinase activity remain relatively constant. Therefore, the D-type cyclins control cell cycle entrance and exit by linking the mitogenic pathway to the core cell cycle machinery.

Unlike cyclin D, the expression of cyclin E is mitogen-independent and dependent on the action of E2Fs after the hyperphosphorylation and inactivation of pRb at the late $G_1$ phase of the cell cycle. However, because pRb represses cyclin E expression, questions remain as to how cyclin E gene expression and cyclin–Cdk2 activity is initially turned on. Once cyclin E levels reach a critical threshold, its levels can be further increased as a result of a positive feedback loop. Active cyclin E:Cdk2 increases degradation of $p27^{Kip1}$ by phosphorylation, which leads to more inactive pRb, free E2F for activation of cyclin E transcription and hence, more active cyclin E:Cdk2.

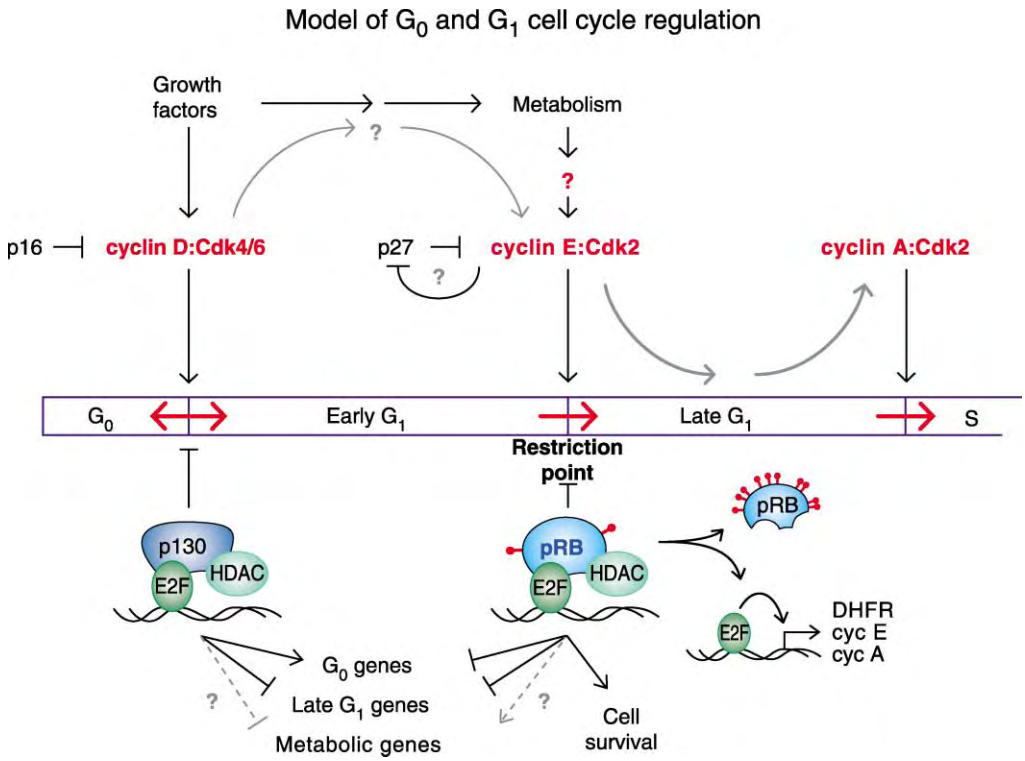



**FIGURE 1** Model of $G_0$ and $G_1$ cell cycle progression. Activation of cyclin D:Cdk4/6 kinases by growth factor stimulation of $G_0$ cells results in increased metabolism or growth and switching of p130:E2F complexes to pRb:E2F complexes. By an unknown mechanism increased metabolism and cell mass leads to activation of cyclin E:Cdk2 complexes at the restriction point resulting in hyperphosphorylation (inactivation) of pRb, release of E2Fs, and activation of late-$G_1$-specific genes involved in DNA synthesis and late $G_1$ cell cycle regulation. Activation of cyclin A:Cdk2 complexes initiates DNA synthesis.

Several mechanisms may contribute to this process. First, $p27^{Kip1}$ in the cyclin E–Cdk2 complex may be sequestered by increased cyclin D–Cdk4/6 complexes. Second, growth factor-activated PI(3)K/Akt pathway may cause the phosphorylation and relocation of the family of forkhead transcription factors into cytosol, which leads to a decrease in the transcription of $p27^{Kip1}$. Furthermore, $p27^{Kip1}$ can be phosphorylated directly by Akt and relocate into cytosol.

The consequence of cyclin D-dependent and cyclin E-dependent kinase activity on pRb also results in differential regulation. It is apparent that cyclin D-dependent kinases are not sufficient for disrupting the pRb-E2F–HDAC complex. Indeed, this is most dramatically supported by the detection of functional pRb–HDAC–E2F complex in $p16^{INK4a}$-deficient cancer cells with deregulated and active cyclin D-dependent kinases. Furthermore, in T cells entering the cell cycle, E2F4 associates with pRb only after cyclin D kinase activity is turned on. In contrast, cyclin E-dependent kinase activity and hyperphosphorylation of pRb are required for disrupting E2F–pRb and HDAC–pRb interactions. Moreover, in addition to pRb family members, cyclin E-dependent kinase phosphorylates target proteins involved in chromatin remodeling, DNA synthesis, centromere functions, further triggering the cell to traverse across the restriction point into late $G_1$ and S phase.

## Cell Growth Regulation

The other aspect of cell cycle regulation is the increase in cell mass or cell growth. In general, cells must grow to a minimum size to be able to divide and generate two daughter cells equal in size to the starting mother cell. Premature entry into S phase results in smaller daughter cells. Therefore, it has been realized, but not well understood, that generally with the exception of some developmental programs, such as early embryonic cell division and endo-reduplication, cell division and cell growth must be coordinated. Although cell growth is often reflected by an increase in cell size, cell growth needs to be defined as an increase in cell mass, or more precisely, the underlying global RNA and protein synthesis capacity, and additional macromolecule synthesis and storage.

How mitotic cells coordinate cell growth and division remains an open question. Some of the key regulators involved in cell cycle regulation also control cell growth. For example, the Myc transcription factor has long been implicated in cell growth regulation in both *Drosophila* and mammalian cells. Besides the gene products involved in cell cycle regulation, such as cyclin D2, Cdk4, cdc25, cyclin E, and cyclin A, many Myc-target gene products are involved in ribosome biosynthesis, protein synthesis, and specific metabolic pathways. Another example of a key cell cycle regulator is pRb. In addition to regulating E2F-dependent gene transcription, pRb can also repress RNA polymerase I- and III-dependent transcription that are responsible for the synthesis of rRNA, various small RNAs such as 5S rRNA, and tRNA. This regulation is mediated by direct interaction of pRb with the upstream-binding factor (UBF) and with the TF-IIIB coactivator, respectively.

On the other hand, cell growth may influence cell division. Understanding of cell growth regulation comes from studying immune suppressant rapamycin. Treatment of both yeast cells and mammalian cells with rapamycin delays cell growth and proliferation. In budding yeast, signaling of the target of rapamycin (TOR), a protein kinase, coordinates nutrient availability, such as carbon source and nitrogen, with cell growth and proliferation. It turns out that the yeast $G_1$/S cyclin Cln3 is both translationally and post-translationally regulated in response to nutrient levels. Cell division occurs when cells reach a certain size. In metazoans, the situation is more complex since nutrient levels are maintained by tissue homeostasis. Not only does mammalian TOR (mTOR) signaling coordinate protein synthesis with glucose and amino acid availability (linked to nutrient sensing signaling pathways), but it also mediates hormonal and mitogenic factor responses. mTOR lies downstream of PI(3) kinase/Akt, although it is currently unclear that they belong to a linear signaling cascade.

PI(3)K/Akt may also activate further downstream targets through tumor suppressor tuberous sclerosis (TSC) complexes. It turns out that the mTOR pathway in conjunction with signaling through the PI(3)K pathway regulates the translational initiation and elongation, ribosome biosynthesis, amino acid import and metabolism. One of the important targets in ribosome synthesis regulated by the PI(3)K/Akt/mTOR pathway is S6 kinase, which phosphorylates ribosomal protein S6. In *Drosophila*, S6 is associated with body size and cell growth. S6 phosphorylation is required for the translation of a group of mRNAs with a 5′ terminal oligopyrimidine tract (5′TOP), including ribosomal protein mRNAs and mRNAs encoding for translation machinery. In addition, the Akt/mTOR pathway regulates protein translation by phosphorylating and inactivating the translation initiation factor 4E-binding protein (4E-BP1). As a result, PI(3)K/Akt and mTOR are linked to translation initiation by regulating the eukaryotic translation initiation factor 4E (eIF-4E), which enhances the translation of essential genes for cell cycle regulation, including cyclin D and $p27^{Kip1}$. Future studies will ultimately resolve how cell growth signaling impinges on and is incorporated into the cell cycle regulatory machinery.

## Future Perspectives

There has been an increased understanding of signal transduction and cell division. A lot of focus has been on protein post-translational modification and gene expression regulation. However, until recently, the importance of cell growth has not been fully appreciated. Clearly there is cross-talk between cell growth and cell cycle regulation. Future studies should fill in the gaps in our understanding of cell growth, cell cycle progression, and their subsequent contributions to neoplastic transformation.

## See Also the Following Articles

Cell Cycle: Control of Entry and Progression Through S Phase • Cell Cycle: Mitotic Checkpoint • Metaphase Chromosome • RNA Polymerase Structure, Bacterial • RNA Polymerase Reaction in Bacteria • RNA Polymerase I and RNA Polymerase III in Eukaryotes

## Glossary

**Cdk inhibitors (CKI)** There are two families of CKI: the Ink4 family proteins (inhibitors of Cdk4: p15INK4b, p16INK4a, p18INK4c, and p19INK4d), which specifically bind monomeric Cdk4 and Cdk6 to inhibit cyclin D-dependent kinase activity and the Cip/Kip family (p21WAF1, p27Kip1, and p57Kip2) that specifically binds and inhibits cyclin:Cdk complexes (cyclin D:Cdk4/6; cyclin E:Cdk2; cyclin A:Cdk2).

**cyclin-dependent kinases (Cdk)** Key activators of the cell cycle. A group of serine/threonine-specific protein kinase complexes composed of a cyclin regulatory subunit and a Cdk catalytic subunit. Activation of the kinase requires cyclin binding to Cdk.

**restriction point** A key cell cycle transition between the early $G_1$ mitogen-dependent phase into the late $G_1$ mitogen-independent phase prior to initiation of DNA synthesis in S phase.

## Further Reading

Abraham, R. T. (2002). Identification of TOR signaling complexes: More TORC for the cell growth engine. *Cell* **111**, 9–12.

Ho, A., and Dowdy, S. F. (2002). Regulation of G1 cell-cycle progression by oncogenes and tumor suppressor genes. *Curr. Opin. Genet. Dev.* **12**(1), 47–52.

Malumbres, M., and Barbacid, M. (2001). To cycle or not to cycle: A critical decision in cancer. *Nat. Rev. Cancer* **1**, 222–231.

Saucedo, L., and Edgar, B. (2002). Why size matters: Altering cell size. *Curr. Opin. Genet. Dev.* **12**(5), 556–565.

Stevaux, O., and Dyson, N. J. (2002). A revised picture of the E2F transcriptional network and RB function. *Curr. Opin. Cell Biol.* **14**, 684–691.

## Biography

Wenge Shi is a postdoctoral Fellow at the Howard Hughes Medical Institute, UCSD School of Medicine. He received his Ph.D. from the University of Wisconsin at Madison.

Steven Dowdy is an Associate Investigator of the Howard Hughes Medical Institute and an Associate Professor of Cellular and Molecular Medicine at UCSD School of Medicine. He received his Ph.D. from the University of California, Irvine and did his postdoctoral fellowship at the Whitehead Institute for Biomedical Research at the Massachusetts Institute of Technology.

# Cell Cycle: Control of Entry and Progression Through S Phase

**Susan L. Forsburg**
*University of Southern California, Los Angeles, California, USA*

**The process of DNA synthesis during S phase requires regulation at multiple levels to ensure that DNA replication is coordinated with overall cell-cycle progression. There are numerous mechanisms to ensure that S phase occurs in a timely and regulated fashion. These ensure the onset of replication occurs in response to the appropriate signals, and they also prevent the re-replication of the genome in a single cell cycle. Much of this regulation occurs by controlling the activation of DNA synthesis at individual origins of replication.**

**The study of S phase and its many levels of control is continuing in many laboratories. This work has benefited from genetic systems such as the yeasts *S. cerevisiae* and *S. pombe*, and the fruit fly *Drosophila*. Biochemical studies primarily in *Xenopus* extracts complement early work that reconstituted the replication apparatus using viral systems and human cells. Given this long and distinguished history, there are inevitably different names for the conserved proteins involved. A listing of the equivalents that are most relevant to this article is provided (Table I).**

## Initiation and Elongation of DNA Replication Origins

The major control of DNA replication occurs at initiation, by regulating assembly and activation at the origins of replication of the multiple proteins of the prereplication complex (preRC). Following initiation, the enzymes of the DNA synthesis machinery take over and extend replication forks to duplicate the genome.

### Origins

DNA synthesis begins at replication origins. Generally, there are many replication origins in each chromosome, to facilitate the timely duplication of the genome. In a few species, the origin is defined by a consensus sequence in the DNA. More commonly, origins are defined by general features of the sequence rather than a linear consensus, and are typically A/T rich and easily unwound. In some cases, origins appear to be distributed not by sequence, but by some sort of spacing mechanism. Not all origins are activated at the same time: while some regions of the genome may undergo initiation early in S phase, others are activated late. Despite these differences, the proteins responsible for replication initiation at individual origins are remarkably similar amongst species.

### Assembly of the preRC

The preRC is assembled by sequential binding of conserved proteins (Figure 1). This occurs at the end of M phase in cycling cells. First, the six proteins of the origin recognition complex (ORC) bind DNA near what will become the initiation site in the replication origin. Next, Cdc6 (called Cdc18 in fission yeast) and Cdt1 bind. These are followed by the six-membered mini-chromosome maintenance (MCM) complex (Mcm2–7). Biochemical studies suggest that once the MCM complex is loaded, the other preRC components are no longer essential for origin initiation. The assembled preRC is now poised for initiation of the origin. The triggers that activate initiation will be discussed later.

### Initiation

Initiation at each origin requires assembly of another set of proteins. It begins with the binding of a protein called Cdc45, which is required for association of the single-stranded DNA-binding protein RPA. This is the essential first step that leads to assembly of primase and DNA polymerase $\alpha$, the initiating polymerase.

Studies suggest that the MCM complex is not only an assembly factor, but plays an active role in elongation. A subset of MCM proteins has *in vitro* helicase activity, and there is some evidence that the MCM proteins are associated with the replication fork. The current model suggests that the MCMs provide an unwinding activity required for replication fork elongation. Curiously, MCMs are extremely abundant proteins, far outnumbering the replication origins, and it remains to be determined why the cell requires so many of them.

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Names of Replication Proteins in Multiple Systems. The same Protein may have Synonyms even in one Species (Indicated by a Slash)**

| *S. cerevisiae* | *S. pombe* | Metazoans | Product |
|---|---|---|---|
| Cdc7 | Hsk1 | Cdc7 | DDK kinase |
| Cdc28 | Cdc2 | Cdk2 and Cdk4 | S phase CDK |
| Cdc6 | Cdc18 | Cdc6 | Activator |
| Mcm2–7 | Mcm2–7 | Mcm2–7 | MCM complex members |
| Orc1–6 | Orp1–6 | Orc1–6 | ORC |
| Cdc45 | Sna41 | Cdc45 | Initiation factor |
| RPA | RPA | RPA | Replication protein A: single-strand DNA-binding protein |
| Dpb11 | Rad4/Cut5 | TopBP1/Mus101 | Possible assembly factor? |
| Tah1 | Cdt1 | Cdt1/Dup | pre-RC factor |
| No homologue[a] | No homologue[a] | Geminin | Cdt1 inhibitor |
| Mec1 | Rad3 | ATR | ATM-related kinase |
| Chk1 | Chk1 | Chk1 | Damage checkpoint kinase |
| Rad53 | Cds1 | Chk2 | Replication checkpoint kinase |
| Drc 1, S1d2 | Drc1 | No homologue[a] | Dpb11 associated factor |
| Sld3 | Sld3 | No homologue[a] | Cdc45 associated factor |
| RFC | RFC | RFC | Replication factor C: PCNA clamp loader |
| Cdc17/Pol1 | Swi7/Pol1 | Pol$\alpha$ | DNA pol $\alpha$ |
| Cdc2/Pol3 | Cdc6/Pol3 | Pol $\delta$ | DNA pol $\delta$ |
| Pol 2 | Cdc20/Pol2 | Pol $\varepsilon$ | DNA pol $\varepsilon$ |
| Cdc44 | Pcn1 | PCNA | Clamp |
| Cdc9 | Cdc17 | Ligase | Ligase |
| Mcm10 | Cdc23 | Mcm10 | Initiation factor |
| Sic 1[b] | Rum 1[b] | P27[b] | Non-homologous CKIs |

[a] Homologue not (yet) identified.
[b] Not related by sequence.

### Elongation

As the leading strand is elongated, a polymerase switch occurs, and the highly processive DNA polymerase $\delta$ assumes the job of DNA synthesis. It is locked into place by a clamp called PCNA, which is loaded onto the DNA by a clamp-loader called RFC. Replication proceeds bi-directionally from the origin, with each leading strand generating a replication fork structure. While DNA polymerase $\delta$ elongates the leading strand, the opposite lagging strand is synthesized in a series of short fragments by DNA polymerase $\alpha$. These Okazaki fragments are joined together by a group of processing enzymes including DNA ligase, to create a single continuous strand. Processive DNA replication may be interrupted if the polymerase encounters lesions in the template, or if it runs out of nucleotide precursors. Checkpoint mechanisms that protect the integrity of the replication fork in these conditions will be discussed below.

Little is known about the resolution of two colliding replication forks from adjacent origins, or the termination of replication. However, studies show that the MCM proteins are dislodged from the chromatin as S phase progresses, and by the conclusion of DNA synthesis they are no longer DNA associated.

## Regulation of Origin Firing by Kinases

Activation of DNA synthesis at the assembled preRC requires the activity of at least two kinases: a cyclin dependent kinase (CDK), and a related kinase called Cdc7 (DDK). Investigators are still examining the molecular effect of these kinases, but a general model for their function has been determined (Figure 1).

### Cdc7 (DDK)

The Cdc7 kinase is essential for initiation of DNA replication. It acts at individual replication origins to initiate DNA synthesis. Its activity requires binding to a regulated subunit, originally identified as Dbf4, which is required for the catalytic Cdc7 subunit to recognize its substrates and targets it to the preRC. Dbf4 is regulated

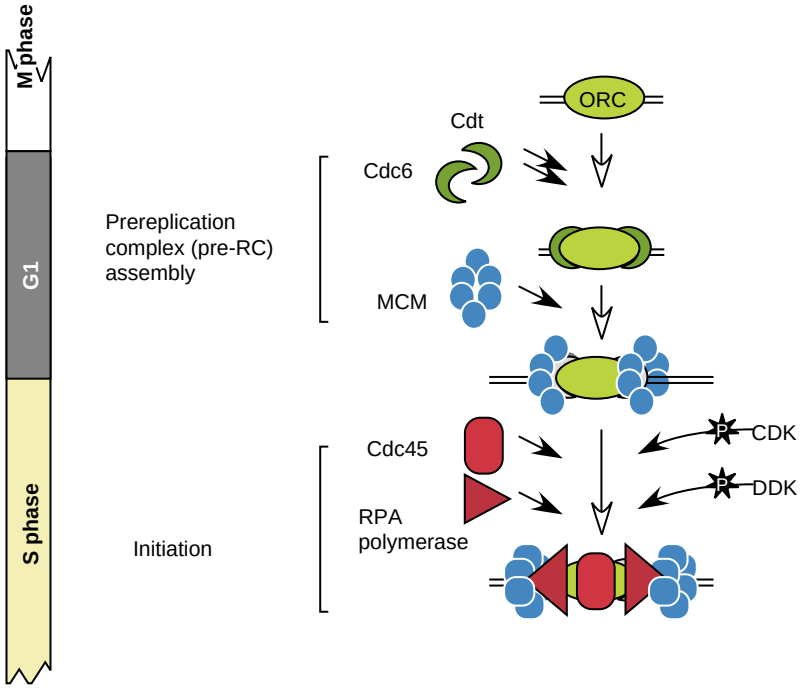



**FIGURE 1** Ordered assembly of the prereplication complex (pre-RC) occurs during late M and G1 phase. Initiation at individual replication origins occurs during S phase. Kinase activity is indicated by stars and results in recruitment of DNA synthesis factors. Positive effects, arrow; Negative effects, bars.

both transcriptionally and posttranslationally, and restricts kinase activity to the S phase of the cell cycle. The Cdc7 kinase is sometimes called a DDK, or Dbf4-dependent kinase, in recognition of its similarity to CDKs. *In vitro* data indicate that Cdc7 kinase can phosphorylate components of the preRC and initiation proteins, including MCM proteins, Cdc45, and DNA polymerase $\alpha$.

A change-of-function mutation in *S. cerevisiae MCM5* was isolated that completely bypasses the requirement for Cdc7 kinase. This led to the suggestion that phosphorylation of the MCM complex by Cdc7 results in a conformational change in the complex that allows Cdc45 to bind to the origin. As described above, Cdc45 binding is a prerequisite for assembly of RPA and primase, and is thought to be limiting for replication. This model has not been confirmed biochemically, but is consistent with genetic data.

### Cyclin-Dependent Kinase

CDKs are the engine of the cell cycle, and their oscillation controls multiple events. In yeasts, a single kinase subunit switches cyclin partners during the cell cycle to provide substrate specificity. In metazoans, multiple kinases combine with multiple cyclins to achieve the same specificity. Studies *in vitro* and *in vivo* investigating replication proteins show that CDK phosphorylates ORC, MCMs, RPA, and DNA polymerase $\alpha$. CDKs play both positive and negative roles in the regulation of DNA replication: while initiation clearly requires CDK activity, the kinase is also required to prevent reinitiation of origins within the same cell cycle (Figure 2).

Recent data from several systems provide an intriguing suggestion that the positive role of CDK in replication initiation operates in a pathway parallel to MCM assembly. In the two yeasts, a conserved protein called Dpb11 (Rad4) interacts with Drc1, which is a known CDK substrate. If Drc1 is not phosphorylated, it cannot bind to Dpb11. Data from *Xenopus* suggest that the Xmus101 protein (related to Dpb11) is required for Cdc45 binding. Together, these studies suggest that two components are required for Cdc45 binding: Cdc7 activation of the preRC, and CDK activation of the Dpb11 pathway. This model is speculative. However, it provides an attractive fail-safe mechanism for the cell to ensure that multiple signals are integrated before it fires a single replication origin, which would significantly reduce the chance of aberrant initiation.

## Coupling Replication to the Cell-Cycle Engine

Activation and inhibition of replication from individual origins is controlled at multiple levels that ultimately depend upon the oscillation of CDK activity during the cell cycle. This oscillation reflects the control of

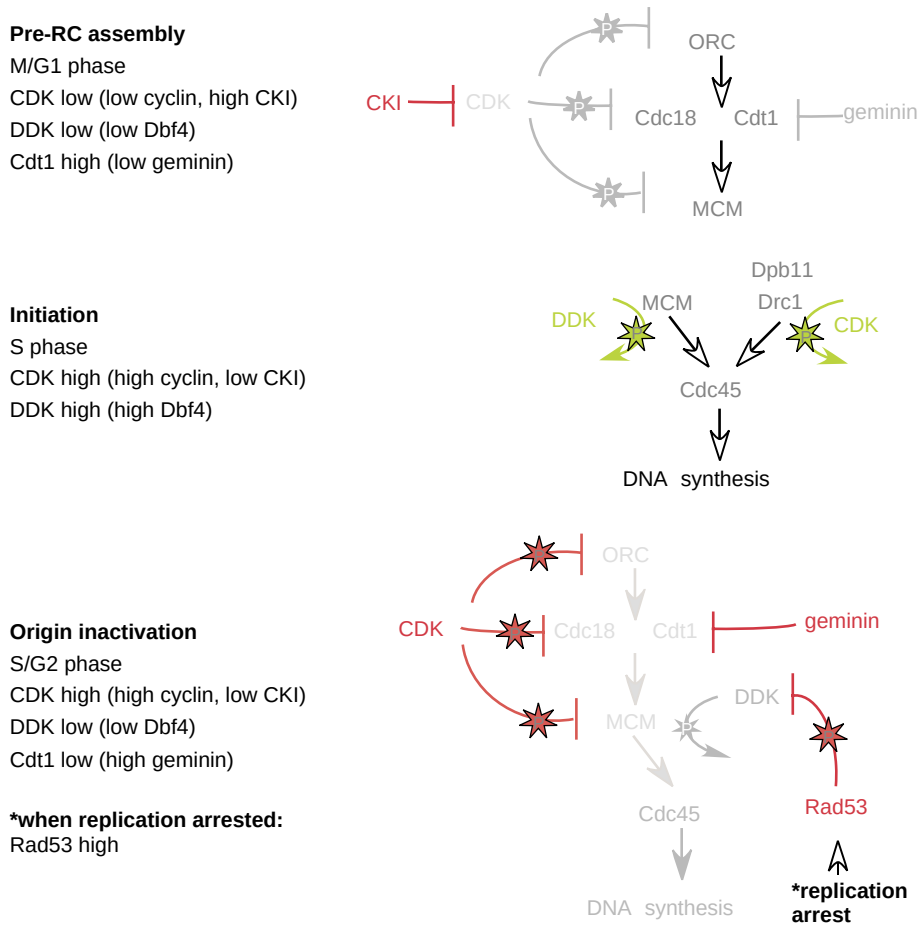



**FIGURE 2** Positive and negative regulation of S phase initiation prevents re-replication. The pre-RC is assembled on origins when CDK activity is low, and reassembly is prevented when CDK activity is high. Origins only fire when both CDK and DDK are active on pre-RCs. Fluctuation in the levels or activity of cyclins, cyclin kinase inhibitors (CKI), Dbf4 protein, and geminin contribute to this regulation. Under conditions when defects in replication block synthesis, Rad53 checkpoint kinase is activated to prevent further origin firing and to protect the structure of active replication forks. Gray text/arrows indicates inactivated pathway components.

the individual cyclins. The DDK subunit Dbf4 is also subject to this regulation (Figure 2).

## CDK Inhibitors and Proteolysis

CDK activity is regulated at multiple levels including phosphorylation. Most obviously, association of the kinase with transiently produced cyclin proteins provides temporal specificity (Figure 2). The cyclins are targeted for destruction by specific ubiquitination complexes at various points in the cell cycle. The Dbf4 subunit of the DDK in yeasts (and probably in larger cells) also oscillates in the cell cycle. It is regulated transcriptionally, and is also a target for ubiquitination during M phase, which restricts its activity to the S phase of the cell cycle. Thus, destruction of proteins in M phase helps maintain the timing of events in S phase.

Specific inhibitor molecules expressed during G1 also modulate CDK activity prior to S phase. These are usually specific to particular cyclin forms of the CDK. These inhibitors keep kinase activity repressed, which allows assembly of a preRC. As cells proceed towards S phase, the increasing levels of cyclin lead to increasing CDK activity which ultimately overcomes the inhibitor. The accumulating CDK phosphorylates its inhibitor, leading to its ubiquitination by an S-phase specific complex that targets the inhibitor for destruction. Although this same general pattern describes most cell types, surprisingly the individual CDK inhibitor molecules in different species such as *S. cerevisiae* Sic1, *S. pombe* Rum1 and human Kip1 are highly diverged, and show no primary sequence homology.

In mammals, Cyclin D/CDK4 phosphorylates and inactivates the Rb protein. Active Rb inhibits E2F, a component of the S-phase-inducing transcription factor; thus Rb needs to be inactivated before S phase can initiate. Because Rb inhibits entry into S phase and the cell cycle, its loss can lead to dysregulation of cell-cycle progression, so it is not surprising that Rb was originally identified as a tumor suppressor.

### Activation of the preRC: Once and Only Once

The negative role of CDKs in preventing reinitiation of DNA replication is dramatically illustrated in fission yeast, in which overproduction of the CDK inhibitor Rum1 results in cells repeating S phase many times, without an intervening mitosis. The CDK is inhibited so it cannot promote mitosis, or prevent reinitiation of replication origins; however, the S-phase-promoting form of the CDK remains active.

The targets of the CDK for this inhibitory activity include several factors at the replication origin. Data suggest that the ORC complex is a CDK target that prevents re-replication. Similarly, Cdc6 protein is tightly regulated so that it is only present in the nucleus at the time of preRC assembly (between mitosis and S phase). It is a CDK substrate, and depending on the system, is either degraded, or exported from the nucleus, in response to phosphorylation. In metazoans, Cdt1 associates with an inhibitor molecule called geminin, which itself is degraded during mitosis to free active Cdt1. Thus, active forms of Cdc6 and Cdt1 are only present at the time of preRC assembly to allow MCM loading. This provides one way to ensure that origins cannot be reactivated in a single cell cycle.

The MCM complex, although present throughout the cell cycle, can only bind at the origin in the presence of Cdc6 and Cdt1. The MCM complex is dislodged during replication, and some subunits are phosphorylated by CDK. In the yeast *S. cerevisiae*, this phosphorylation results in the active export of MCMs from the nucleus as S phase proceeds. The dislodged proteins remain in the nucleus in other species, but are unable to bind the chromatin. The MCMs cannot regain access to the origin (or in *Saccharomyces*, the nucleus) until CDK levels fall at the end of mitosis, and Cdc6 and Cdt1 are active again in the next G1 phase.

This sequential requirement for origin binding allows tight cell-cycle regulation of initiation. Low CDK activity allows pre-RC assembly. High CDK activity allows origin firing, but prevents reassembly of initiation factors. The CDKs thus play a dual role by both promoting initiation and preventing re-replication at individual origins.

## Genome Integrity during S Phase

The assembly and elongation of a replication fork involves nicks and unwinding, generating fragile regions in the chromosome that may be prone to breakage. Replication does not proceed all in one piece, but involves pauses and stops along the way. Thus, the cell must have a sensitive quality control mechanism to prevent damage from occurring, and to respond appropriately if it does occur. The response prevents mitosis (by inhibiting the mitotic CDK), and repairs the damage (by activating repair proteins). Components of the replication checkpoint pathways are responsible for processing these signals and generating the correct response. Proteins required for repair via recombination are also likely to be important components for quality control during DNA replication.

Data suggest that the Rad53/Cds1/Chk2 kinase is a crucial component of the response to replication defects in all eukaryotes. In a few species, cells can survive loss of this kinase, but even these survivors show genome instability and sensitivity to damaging agents when it is missing. Some experiments suggest that the Rad53/Cds1/Chk2 kinase regulates the timing of origin activation even in normal cells, by inhibiting DDK activity. But its best-understood function is in response to replication arrest early in S phase. The kinase maintains the structure of the assembled replication fork and promotes repair, by phosphorylation of substrates in the recombination-repair pathway. It also prevents initiation from late origins during replication arrest by inhibiting DDK activity.

## Regulating Chromosome Structure during S Phase

The events of S phase are intimately linked to chromosome structure. Although the mechanism is not clear, it is known that cohesion between sister chromatids is established during S phase, as the sister chromatids are generated. Cohesion is essential for proper segregation of the chromosomes during mitosis. It prevents chromosome loss, contributes to centromere structure, and helps orient the chromatids to the opposite spindles. Whether the passing replication fork activates the cohesin proteins, or they are activated by the S-phase kinases, remains to be determined.

Chromatin assembly is also affected during S phase. The cell must assemble its newly duplicated DNA into nucleosomes and higher-order chromatin structures. The mechanisms for this are also not well understood, but are likely to involve conserved protein complexes activated during DNA replication. Chromatin structure may in turn regulate S-phase timing. Recent studies suggest that modification of histones by acetylation may help determine the timing of firing for individual origins.

## Conclusions

Events during S phase have profound effects on chromosome biology throughout the cell cycle. The replication fork has an enormous capacity to remodel chromatin.

In addition, activation of specific kinases only during S phase can provide a temporal link between DNA replication and modification of other proteins. Clearly, investigators are only beginning to trace these links. The study of S-phase regulation of multiple chromosome events will expand for years to come.

## See Also the Following Articles

DNA Ligases: Mechanism and Functions • DNA Ligases: Structures • DNA Replication Fork, Bacterial • DNA Replication: Initiation in Bacteria • Recombination-Dependent DNA Replication

## Glossary

**CDK** Cyclin-dependent kinase, a family of kinases that requires binding to a regulatory subunit called a cyclin to be active against exogenous substrates.

**CKI** Cyclin-kinase inhibitor.

**DDK** DBF4-dependent kinase Cdc7, required for replication initiation.

**origin** Site of replication initiation in the genome; usually intergenic, but often has no defining sequence.

**preRC** Prereplication complex assembled before origin firing comprises ORC, Cdc6, Cdt1, and MCM proteins.

## Further Reading

Bell, S. P., and Dutta, A. (2002). DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71**, 333–374.

Diffley, J. F., and Labib, K. (2002). The chromosome replication cycle. *J. Cell Sci.* **115**, 869–872.

Kelly, T. J., and Brown, G. W. (2000). Regulation of chromosome replication. *Annu. Rev. Biochem.* **69**, 829–880.

Lei, M., and Tye, B. (2001). Initiating DNA synthesis: From recruiting to activating the MCM complex. *J. Cell Sci.* **114**, 1447–1454.

Takisawa, H., Mimura, S., and Kubota, Y. (2000). Eukaryotic DNA replication: From pre-replication complex to initiation complex. *Curr. Opin. Cell Biol.* **12**, 690–696.

## Biography

Susan L. Forsburg is an Associate Professor of Molecular and Computational Biology at the University of Southern California in Los Angeles. Her research investigates the initiation of DNA replication and regulation of chromosome dynamics in the fission yeast *Schizosaccharomyces pombe*. Prior to joining USC in 2004 she was on the facility at the Salk Institute in La Jolla, California. She received her Ph.D. from MIT, and carried out postdoctoral training at the Imperial Cancer Research Fund at Oxford University.

# Cell Cycle: DNA Damage Checkpoints

**Jean Y. J. Wang**
*University of California, San Diego, La Jolla, California, USA*

**DNA damage activates cell cycle checkpoints to prevent replication and segregation of damaged genome. DNA damage also inhibits cellular differentiation, activates apoptosis, and induces premature senescence. Defects in these checkpoint responses to DNA damage contribute to tumor progression, resistance to radiation/chemotherapy, and the development of degenerative diseases.**

## Overview

The orderly progression through a cell division cycle is controlled by a series of checkpoints. Two fundamental checkpoints, i.e., replication checkpoint and spindle checkpoint, operate in every round of cell cycle (Table I). The replication checkpoint monitors progress of DNA replication; it prevents entry into mitosis until replication is completed. The spindle checkpoint monitors attachment of chromosomes to the mitotic spindles; it prevents segregation of sister chromatids until they are properly aligned on the metaphase plate. Under normal physiological conditions of abundant energy and nutrients, these checkpoints are experimentally invisible. Perturbation in the cellular environment can prolong the operation of these two checkpoints and/or activate additional checkpoints, leading to an observed inhibition of cell cycle progression.

This article discusses three distinct cell cycle checkpoints activated by DNA damage: the G1/S checkpoint, the intra-S checkpoint and the G2/M checkpoint (Figure 1). The G1/S checkpoint inhibits S-phase entry in G1 cells that have not yet committed to DNA replication. The intra-S checkpoint prevents initiation of DNA replication at origins that have not yet been activated. The G2/M checkpoint inhibits entry into mitosis. DNA damage also delays differentiation, activates programmed cell death, or induces premature senescence (Figure 2). The inhibition of cell cycle progression or differentiation is reversible, allowing resumption of cell proliferation and differentiation after DNA repair. Apoptosis and premature senescence are irreversible, most likely triggered by the accumulation of persistent lesions in the genomic DNA.

## Cell Cycle Checkpoints Activated by DNA Damage

### G2/M Checkpoint

The G2/M checkpoint is activated by a variety of DNA lesions, including base modification, cross-linking, and strand break. The sister chromatids in G2 cells are advantageous to DNA repair. Thus, G2 arrest serves two important purposes: to promote the repair of DNA lesions and to prevent mitotic catastrophe caused by segregating damaged DNA. The G2/M checkpoint causes G2 arrest by blocking mitosis (Table I).

#### Inhibition of Mitosis

In all eukaryotic cells, mitosis requires the cyclin-dependent protein complex of Cdc2/M-cyclin (also known as MPF). This MPF complex is formed throughout S and G2, but held in a latent state through phosphorylation of a threonine and a tyrosine in the N-terminal ATP-binding lobe of Cdc2. The G2/M checkpoint prevents dephosphorylation of Cdc2, holding MPF in the inactive state, and thereby prolongs G2 phase of the cell cycle (Figure 1).

DNA damage maintains the inhibitory phosphorylation of Cdc2 by several mechanisms. Dephosphorylation of Cdc2 at the two inhibitory sites requires a dual specificity phosphatase encoded by the highly conserved Cdc25 gene. DNA damage causes an inhibition of Cdc25 phosphatase through the Chk1 kinase, which is also conserved in all eukaryotes. Chk1 phosphorylates Cdc25 to inhibit its activity and its access to MPF. Sequestration of phosphorylated Cdc25 requires the adaptor protein 14-3-3, another function that is conserved through evolution. The Cdc25 phosphatase is further controlled by DNA damage

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Summary of Cell Cycle Checkpoints**

| Checkpoint | Trigger | Target of inhibition |
|---|---|---|
| Replication checkpoint | Cell cycle intrinsic | MPF |
| Spindle checkpoint | Cell cycle intrinsic | Sister chromatin separation |
| G2/M checkpoint | DNA lesions | MPF (Sister chromatin separation) |
| Intra-S checkpoint | DNA lesions (stalled replication forks) | Origins of replication |
| G1/S checkpoint | DNA lesions | SPF |

through the inhibition of polo-like kinase 1 (Plk1), which activates Cdc25. DNA damage also maintains the activity of Wee1, the conserved kinase that phosphorylates Cdc2 at the inhibitory tyrosine site. Therefore, DNA damage targets several regulators of MPF to keep it in the latent state and thereby blocking the entry into mitosis.

### Replication Block Also Inhibits Mitosis

The cell cycle intrinsic replication checkpoint also controls the activity of MPF (Table I). Experimentally, replication checkpoint function is measured by the inhibition of MPF under conditions when DNA replication is blocked. Hydroxyurea (HU), an inhibitor of nucleotide biosynthesis, is commonly used to reveal the replication checkpoint. In the fission yeast, HU-induced inhibition of MPF requires Cds1 kinase, another checkpoint protein conserved in all eukaryotes. The budding yeast contains two Cds1-related kinases: RAD53 and DUN1. The mammalian homologue of Cds1 is commonly known as the Chk2 kinase. Similar to Chk1, Cds1 (Chk2) kinase also inhibits Cdc25 to keep MPF in the inactive state. DNA damage-induced G2 arrest is therefore triggered by a mechanism that overlaps in part with the cell-cycle-intrinsic replication checkpoint.

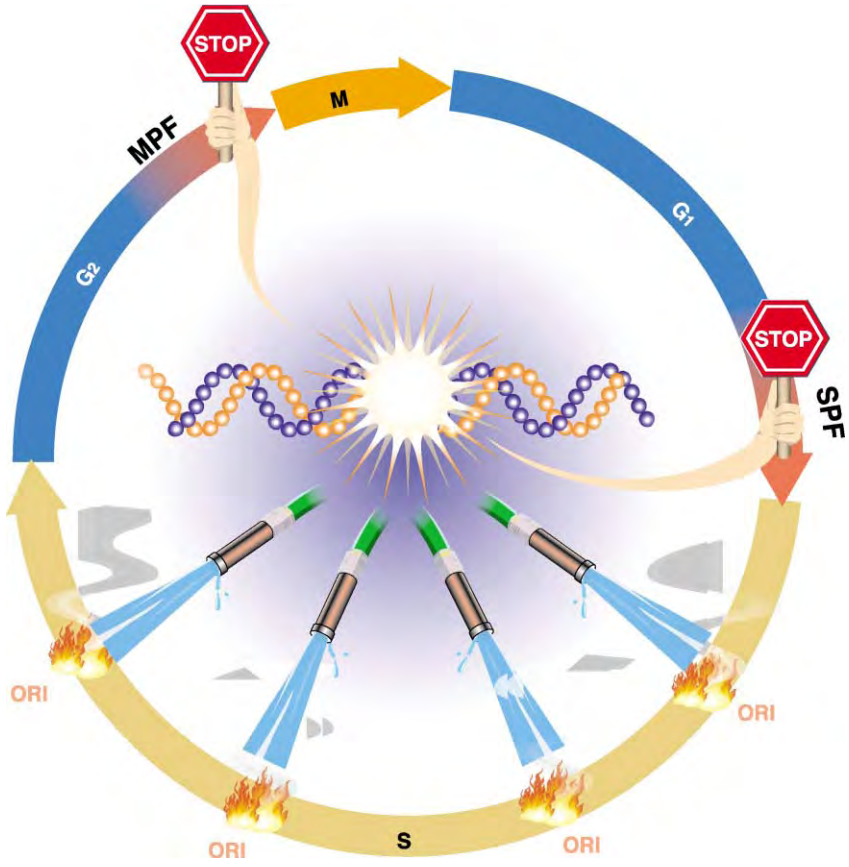



**FIGURE 1** DNA damage checkpoints. DNA lesions activate three distinct cell cycle checkpoints. The G1/S checkpoint prevents G1 cells from entering S phase by inhibiting the function of S-phase-promoting factor (SPF). The intra-S checkpoint inhibits the origins of replications (ORI). The G2/M checkpoint prevents G2 cells from entering M phase by maintaining the M-phase-promoting factor (MPF) in its latent, inactive state.

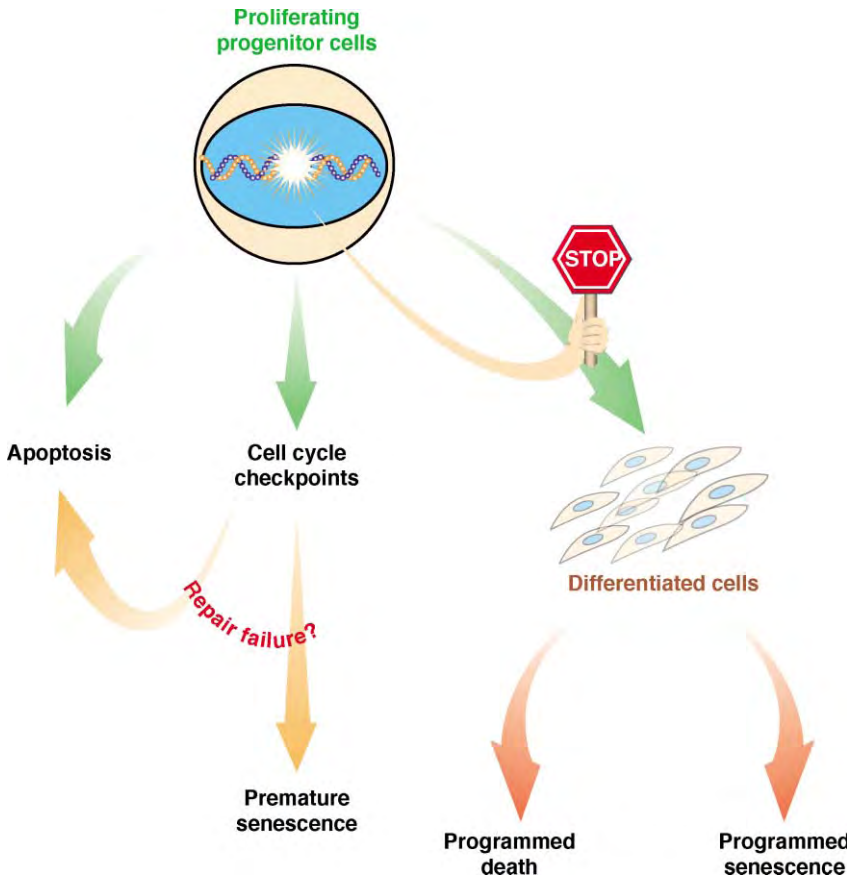



**FIGURE 2** DNA damage responses. Cell cycle checkpoints are among several different cellular responses to DNA damage. In some cell types, DNA damage triggers a rapid death response, through the activation of the cellular suicide program (apoptosis). DNA damage also halts differentiation, preventing the global reprogramming of gene expression until lesions are repaired. Death and senescence are preprogrammed for different types of terminally differentiated cells. Interestingly, DNA damage can also cause death or senescence as a delayed response that is likely to be triggered by failure in DNA repair. The choice between apoptosis or premature senescence is not determined by the damage alone, but also dependent on cell type and developmental lineage.

### Overlapping Function of Chk1 and Chk2 Kinases in Blocking Mitosis

In fission yeast, Chk1 is required for DNA damage to cause G2 arrest; whereas Cds1 is required to prevent premature mitosis when replication is blocked. In the budding yeast, RAD53 is required for both the replication checkpoint and the damage-induced G2/M checkpoint. Unlike the fission yeast, the budding yeast does not arrest in G2 following DNA damage. Instead, the budding yeast arrests at the metaphase–anaphase transition, unable to separate sister chromatids. Thus, DNA damage activates a G2/M-phase checkpoint that overlaps in part with the spindle checkpoint in the budding yeast. Whether DNA damage also maintains the cohesion of sister chromatids in other eukaryotic cells awaits further investigation. In mammalian cells, current results suggest Chk1 and Chk2 to have redundant and overlapping functions in DNA damage-induced G2 arrest. In mouse embryonic stem (ES) cells, elimination of Chk1 but not Chk2 causes mitotic catastrophe, suggesting Chk1 to have an essential role in preventing premature mitosis in every round of the ES cell cycle.

## Intra-S Checkpoint

The intra-S checkpoint is activated by DNA lesions that impede the progress of replication forks. The eukaryotic genomes are divided into replicons, each with an origin of replication. During G1 phase, origins become licensed for replication. Not all of the licensed origins are activated at the onset of S phase. Instead, they are activated in stages; some of them activated early, others late in S phase. When ongoing replication forks are stalled due to DNA lesions, intra-S checkpoint is activated to prevent initiation at licensed origins that have not yet been activated (Table I). Stalled replication, as discussed above, also prevents the activation of MPF; this is known as the replication checkpoint. Therefore, stalled replication forks can cause the inhibition of

replication origins (intra-S checkpoint), or the inhibition of MPF (replication checkpoint) (Table I).

### Intra-S Checkpoint Defect in *Ataxia telangiectasia*

In mammalian cells, defect in the intra-S checkpoint was first described decades ago as radiation-resistant DNA synthesis (RDS) in cells isolated from patients suffering from the genetic disease *Ataxia telangiectasia* (AT). Exposure of normal human fibroblasts to ionizing radiation causes a transient inhibition of DNA synthesis due to the inhibition of replication origins. Thus, ionizing radiation (IR), which generates single-stranded and double-stranded breaks, can activate the intra-S checkpoint. Fibroblasts from AT patients continue to synthesize DNA irrespective of IR, i.e., exhibiting the phenotype of RDS. Therefore, RDS results from a defect in the intra-S checkpoint; and *ATM*, the gene mutated in AT, is required for IR to activate the intra-S checkpoint in mammalian cells.

### Inhibition of Replication Origins

The target of inhibition by the intra-S checkpoint is the licensed origins of replication. Licensing of replication is a complicated process involving the orderly assembly of a large protein complex called the pre-RC (replication complex) during G1. The pre-RC is then converted to pre-IC (initiation complex) in late G1. Activation of the pre-IC initiates the onset of S phase. The formation and activation of pre-IC requires two types of protein kinase complexes: the Cdk/S-cyclin complex and the Cdc7/Dbf4 kinase complex, both are conserved through evolution. In the budding yeast, inhibition of late origins during S phase requires the RAD53 kinase and involves the inhibition of Cdc7/Dbf4. In mammalian cells, the IR-induced and ATM-dependent intra-S checkpoint requires the Chk2 kinase, which phosphorylates Cdc25A to promote its degradation. Mammalian cells contain three Cdc25 genes: *A*, *B*, and *C*. Cdc25C dephosphorylates and activates the Cdc2/M-cyclin complex (MPF), whereas Cdc25A dephosphorylates and activates the Cdk/S-cyclin complex (S-phase-promoting factor, SPF). Degradation of Cdc25A, therefore, interferes with the formation and activation of pre-IC, leading to the inhibition of replication initiation. Whether the mammalian Cdc7/Dfb4 complex is also a target of the mammalian intra-S checkpoint remains to be determined. The Cdk/S-cyclin and Cdc7/Dbf4 kinases may not be the only targets of the intra-S checkpoint. Ongoing studies in the yeast model system have suggested several intra-S checkpoint pathways. Therefore, molecular mechanisms of intra-S checkpoint have yet to be fully elucidated.

## G1/S Checkpoint

The G1/S checkpoint is activated by DNA damage in cells that have not yet initiated replication, and its function is to prevent the onset of S phase. The G1/S checkpoint inhibits Cdk/G1–cyclin to prolong the G1 phase. The G1/S checkpoint also inhibits Cdk/S–cyclin to prevent the onset of S phase, thus arresting cells in G1.

### The p53-Dependent G1 Arrest

The most well-known mechanism for DNA damaged-induced G1 arrest is that mediated by the tumor-suppressor protein p53 in mammalian cells. DNA damage stabilizes the p53 protein and activates its transcription function. One of the p53 target genes is p21Cip1, a heat-stable inhibitor of Cdk/cyclin. The p53-dependent up-regulation of p21Cip1 causes inhibition of Cdk2/cyclin-E and Cdk2/cyclin-A to block S-phase entry. This p53-mediated G1 arrest is a slower response, because up-regulation of p21Cip1 requires transcription and new protein synthesis. The p53 gene is conserved in metazoan. However, the *Drosophila* p53 does not stimulate the expression of p21Cip1. Therefore, the p53/p21Cip1 pathway is not a conserved mechanism for DNA damage to cause G1 arrest.

The p53 protein belongs to a family of transcription factors with similar DNA binding specificity. The other two members, p63 and p73, are also activated by DNA damage. Together, this family of transcription factors may induce G1-arrest, premature senescence, or apoptosis in damaged cells (Figure 2). Premature senescence describes the phenotype of long-term arrest in G1, S, or G2. This long-term arrested state requires the RB-family of pocket proteins, which repress the expression of E2F-regulated cell cycle genes. DNA damage-induced apoptosis requires the functions of p53 and its related p63 and p73 proteins. The current model proposes that this family of transcription factors regulates the expression of pro-apoptotic proteins to induce cell death. At present, we do not understand how a damaged cell chooses between premature senescence and apoptosis through p53-family proteins. Choice between these two irreversible cell fates appears to be determined by cell types and developmental lineage.

### Inhibition of S-Phase Entry

The up-regulation of p21Cip1 is not the only way to inhibit G1/S transition by DNA damage. In late G1 cells, Cdk2 is in complex with cyclin-E, and Cdk2/cyclin-E is required for the initiation of DNA replication. DNA damage can cause the inhibition of Cdk2/cyclin-E by two mechanisms: the up-regulation of p21Cip1 and the degradation of Cdc25A. As discussed above,

degradation of Cdc25A contributes to the inhibition of pre-IC in S phase cells. The inhibition of pre-IC can also cause G1 arrest if it occurs prior to the onset of S phase. Current evidence suggests that p21Cip1 only prevents the onset of S phase but does not contribute to the intra-S checkpoint. The degradation of Cdc25A, however, can inhibit S-phase entry and origin activation within the S phase.

### Delay in G1 Progression

In the budding yeast, a short-lived G1 delay is observed in response to DNA damage. This G1 delay requires RAD53 and involves the inhibition of G1-cyclin (yeast CLN) expression. The mammalian G1-cyclins are the D-type cyclins, which form complex with Cdk4 or Cdk6. Degradation of cyclin D1 has been observed in response to DNA damage. The D-type cyclins promote G1 progression but they are dispensable for DNA replication. Loss of D-type cyclins alone is not likely to block S-phase entry, but may contribute to a delay in G1 progression.

## DNA Damage Signal Transduction

The G1/S, intra-S, and G2/M checkpoints are terms to describe the distinctive effects of DNA damage on the cell cycle. These descriptive terms, however, do not imply distinctive molecular mechanisms. As discussed above, these cell-cycle effects can result from a common biochemical mechanism. For example, negative regulation of Cdc25 phosphatase can inhibit the onset of DNA replication in G1, prevent origin firing in S phase, or arrest cells in G2. Indeed, current evidence suggests DNA damage checkpoints are the effects of a common signal transduction network, the core of which is conserved in all eukaryotic cells. The conserved core components of this DNA damage-signaling network are listed in Table II. This list is far from complete, because the workings of this network are still under investigation.

### The 9–1–1 Complex

Functioning at the top of the conserved signaling network is a PCNA-like protein complex composed of three proteins forming a heterotrimer that resembles the PCNA homotrimeric DNA-clamp. This PCNA-like complex is conserved from yeast to man. Based on the fission yeast gene names (RAD9, Rad1, Hus1), a unifying term has recently been coined to describe this trimer as the 9–1–1 complex. The 9–1–1 complex is loaded onto DNA by a protein (fission yeast Rad17) that resembles a subunit of the RFC (replication factor C). The normal RFC loads PCNA at the origin of replication. Rad17 is a component of a special RFC that loads the 9–1–1 complex onto damaged DNA. Together, Rad17-RFC and 9–1–1 function as a sensor of DNA damage. The 9–1–1 complex may provide a molecular platform for the assembly of DNA damage signaling complex.

**TABLE II**

**Conserved Core Components in DNA Damage Signal Transduction**

| Name | Biochemical activity | Role in DNA damage signaling |
|---|---|---|
| Rad17-RFC | Loading the 9–1–1 clamp onto damaged DNA | Sensor |
| 9–1–1 | Heterotrimeric DNA clamp | Sensor |
| ATR, ATM, MEC1 (*S.c.*), Rad3 (*S.p.*) | Protein kinase of the PI3-kinase superfamily | Master switch |
| BRCA1, p53-BP1, MDC1, RAD9 (*S.c.*), Crb2 (*S.p.*) | Varied | Adaptor |
| Chk1 | Protein kinase | Effector |
| Chk2, RAD53 (*S.c.*), Cds1 (*S.p.*) | Protein kinase with FHA domain | Effector |

*S.c.: Saccharomyces cerivisiae*; *S.p.: Schizosaccharomyces pombe.*

### Protein Kinase of the PI3K Family

The master switch in DNA damage signaling is a protein kinase of the PI3-kinase superfamily. In the budding yeast, *mec1* gene encodes this master protein kinase. In the fission yeast, *rad3* encodes a similar master protein kinase. In mammalian cells, ATR is likely to be the functional homologue of the yeast MEC1, Rad3 protein kinase. The MEC1, Rad3, and ATR kinase each associates with an accessory factor, encoded by the budding yeast Ddc2, fission yeast Rad23 and mammalian ATRIP, respectively. The MEC1/Ddc2, Rad3/Rad26 or ATR/ATRIP complex each associates with damaged DNA, in parallel with the Rad17-RFC/9–1–1 sensor. The MEC1, Rad3 and ATR kinase then phosphorylate downstream effectors to propagate the damage signals.

In mammalian cells, the ATM kinase, another member of the PI3K-family, also plays an important role in DNA damage signaling. As discussed above, ATM is required for IR to activate the intra-S checkpoint. *ATM* is not an essential gene. *Atm*-knockout mice are born, and they exhibit many of the phenotypes of AT patients. By contrast, *ATR* is essential for life. *Atr*-knockout causes early embryonic lethality in mice. Conditional knockout of *Atr* causes lethality

within one round of cell cycle, suggesting ATR to control cell cycle intrinsic regulatory processes.

## Chk1 and Chk2 Kinases

Downstream of the master switch kinase is a network of substrates with varying degree of evolutionary conservation. The most highly conserved substrates with essential functions in DNA damage signaling are the Chk1 and Chk2 kinases. The fission yeast Chk1 is required for DNA damage to prevent the activation of MPF. This function is conserved for the metazoan Chk1 kinase. The mammalian Chk2 is homologous to the fission yeast Cds1, and the budding yeast RAD53 and Dun1. The Chk2-family of kinases is distinguished by the FHA domain, which is not found in Chk1. The FHA domain preferentially interacts with peptides that contain a phospho-threonine residue. The FHA domain in Chk2 mediates phosphorylation-dependent protein-protein interaction and plays a critical role in the regulation of Chk2 kinase activity. The Chk1 and Chk2 kinases are directly phosphorylated by ATR/ATM kinases in mammalian cells. Phosphorylation is necessary, although not sufficient, for activation of the Chk1 and Chk2 kinase activity.

## BRCT-Containing Adaptor Proteins

The master switch kinase also phosphorylates another class of proteins, which have been postulated to function as "adaptors" in DNA damage signal transduction. The founding member of this class of adaptor proteins is the budding yeast RAD9 gene product. The RAD9 protein contains a pair of BRCT domains at its C terminus. RAD9 is phosphorylated by the PI3 kinase; and phosphorylated RAD9 has an essential role in activating RAD53 kinase. In the fission yeast, Crb2 contains a pair of BRCT domains at its C terminus and it is required for DNA damage to activate Chk1 kinase. In mammalian cells, at least three proteins have exhibited structural and functional characteristics similar to RAD9. These are BRCA1 (breast cancer associated 1), p53-BP1, and MDC1 (also known as Kiaa0170). Each of these three proteins are phosphorylated in response to DNA damage and each plays important roles in activating DNA repair or cell cycle checkpoints. Whether these BRCT proteins have specific or redundant functions in DNA damage signal transduction is presently unknown.

## Other Transducers

DNA damage signal transduction involves many more proteins than those summarized in Table II. A number of downstream effectors in this signal transduction network are not conserved through evolution and not included in Table II. A good example is the p53 tumor-suppressor protein. As discussed earlier, p53 is conserved in metazoan. The conserved biological effect of p53 is to activate apoptosis in response to DNA damage. In vertebrates, p53 can additionally activate p21Cip1 to cause G1 arrest. The conserved core of the DNA damage signal transduction network, therefore, recruits non-conserved effectors to tailor the biological responses. If so, studies of the conserved core alone may not elucidate all the damage response pathways. Instead, each response to DNA damage may have to be investigated in an appropriate experimental system to identify the specific downstream effectors for that biological response.

## See Also the Following Articles

DNA Base Excision Repair • DNA Damage: Alkylation • DNA Mismatch Repair and the DNA Damage Response • Mitosis

## Glossary

**ATM** A large-molecular-weight protein encoded by the gene that is mutated in the human disease AT. AT patients suffer from cerebella degeneration, extreme sensitivity to ionizing radiation, increased cancer risk, and sterility. *Atm*-knockout mice exhibit most of the AT phenotypes, except cerebella degeneration. ATM contains a PI3-kinase homology domain and its function is required for IR to activate the DNA damage checkpoints.

**ATR** ATM and Rad3 related, a protein with a PI3-kinase homology domain. ATR has an essential function in mammalian cells, and is required for the activation of replication, G2/M, and intra-S checkpoints.

**BRCT** A modular protein–protein interaction domain conserved through evolution and found in proteins required for checkpoint activation or DNA repair.

**Cdk** Cyclin-dependent protein kinase. Cdk forms complex with and is activated by cyclin. Cdk1 is also known as Cdc2 kinase.

**Chk1** Checkpoint kinase-1, first identified in the fission yeast and conserved through evolution. DNA damage activates Chk1 to maintain MPF in the latent, inactive state. Chk1 is not an essential gene in yeasts, but Chk1-knockout mouse ES cells die from mitotic catastrophe.

**Chk2** Checkpoint kinase-2, first identified in the fission yeast as the Cds1 kinase and conserved through evolution. The budding yeast contains two Chk2-like kinases: RAD53 and DUN-1. Germline mutation of the *CHK2* gene is found in a fraction of patients with the Li–Fraumeni cancer syndrome, which can also result from germline mutation of the p53 tumor-suppressor gene.

**Cyclins** Activators of cyclin-dependent kinases. Cyclins are expressed and degraded periodically through the cell cycle.

**FHA** A modular protein–protein interaction domain conserved through evolution. FHA domain binds peptides with phosphorylated-threonine. However, binding to unphosphorylated peptide can also occur. FHA domain is found in a variety of proteins with diverse functions, including those involved in DNA damage signal transduction.

**MPF** M-phase-promoting factor, a protein kinase composed of a catalytic subunit Cdc2 and a regulatory subunit, M-phase cyclin.

**premature senescence** A prolonged growth arrest induced by DNA damage. The arrest can occur in G1, S, or G2 and is mediated by the repression of cell cycle genes.

**SPF** S-phase promoting factor. In the context of this article, SPF denotes the function of Cdk2, activated by the mammalian E-type or A-type cyclins.

## Further Reading

Bork, P., Hofmann, K., Bucher, P., Neuwald, A. E., Attschul, S. F., and Koonin, E. U. (1997). A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. *Faseb J.* **11**, 68–76.

Durocher, D., and Jackson, S. P. (2002). The FHA domain. *FEBS Lett.* **513**, 58–66.

Melo, J., and Toczyski, D. (2002). A unified view of the DNA-damage checkpoint. *Curr. Opin. Cell. Biol.* **14**, 237–245.

Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002). Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet.* **36**, 617–656.

Rouse, J., and Jackson, S. P. (2002). Interfaces between the detection, signaling, and repair of DNA damage. *Science* **297**, 547–551.

Yang, A., Kaghad, M., Caput, D., and McKeon, F. (2002). On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet.* **18**, 90–95.

Zhou, B. B., and Elledge, S. J. (2000). The DNA damage response: Putting checkpoints in perspective. *Nature* **408**, 433–439.

## Biography

Jean Y. J. Wang is a Professor in the Division of Biological Sciences at the University of California, San Diego. Her principal research interest is to understand mammalian cell growth control. Her research has been focused on elucidating the role of retinoblastoma tumor suppressor protein, Abl tyrosine kinase and p73 in regulating differentiation and apoptosis. She holds a Ph.D. in biochemistry from the University of California, Berkeley. She was also a postdoctoral fellow at the Massachusetts Institute of Technology Cancer Center.

# Cell Cycle: Mitotic Checkpoint

**Tim J. Yen**
*Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA*

**The mitotic checkpoint is a fail-safe mechanism that evolved to ensure that cells with even a single unaligned chromosome do not exit mitosis to produce aneuploid cells. The mitotic checkpoint solves a inherent problem that arises because of the stochastic nature by which chromosomes attach to the spindle. At the onset of mitosis, chromosomes are randomly distributed throughout the cell so that not all chromosomes will achieve alignment at the spindle equator at the same time. Chromosomes located in the center of the spindle will rapidly establish bipolar attachments due to their higher frequency of encounters with microtubules. By contrast, chromosomes located near a pole will rapidly attach to that pole but attachment to the opposite pole will take more time given the lower frequency that it will find the rare microtubule that originates from the opposite pole. As the kinetochore is the structure on chromosomes that establishes connections with spindle microtubules, the checkpoint monitors this site to determine if chromosomes are properly attached to the spindle and whether it has achieved alignment.**

**The goal of this article is to discuss current models that explain how the checkpoint monitors kinetochore functions and how a localized defect that is restricted in space can alter the global biochemical status of a cell.**

## The Mitotic Checkpoint Monitors Kinetochores

The checkpoint is able to sense the status of chromosome alignment by monitoring microtubule occupancy and tension (or the lack thereof) at kinetochores. Once a defect is detected, the kinetochore generates an inhibitory signal to block the onset of anaphase. Early studies showed that a single unattached kinetochore is sufficient to arrest cells in mitosis. Thus, the mitotic checkpoint functions much like a signal transduction cascade where a defective kinetochore generates a signal that must be amplified throughout the cell to inhibit its targets. The discovery of the molecular components of the mitotic checkpoint has shed considerable light into how the mechanical activities at the kinetochore can regulate mitotic progression.

## Molecular Components of the Mitotic Checkpoint

The mitotic checkpoint is specified by six evolutionarily conserved genes, BUB1, BUB3, MAD1, MAD2, MAD3, and MPS1. All of these genes are essential as inactivation of any single gene prevents cells from delaying mitosis in the presence of unaligned chromosomes. BUB1 and MPS1 are protein kinases, while the biochemical activities of the remaining proteins are unknown. It appears that MAD3 in metazoans has evolved into BUBR1, a third protein kinase of the mitotic checkpoint. It is interesting that BUBR1 may have co-evolved with CENP-E, a kinetochore motor whose activity is thought to be monitored by BUBR1 and is also only present in metazoans. With the identification of checkpoint genes, the outstanding question is how their gene products interact at the molecular and biochemical levels to specify the signaling pathway that links a defective kinetochore to inhibition of the anaphase promoting complex (APC).

### Monitoring Microtubule Occupancy and Tension at Kinetochores

The discovery that mitotic checkpoint proteins preferentially localize to unattached kinetochores suggested their roles in monitoring kinetochore activities and generating the "wait anaphase" signal. In this regard, the role of the MAD2 checkpoint protein at kinetochores has been extensively characterized. Quantitative studies have shown that the staining intensity of MAD2 at unattached kinetochores can be nearly 100-fold higher than that detected at kinetochores that are fully saturated with microtubules. In contrast, the intensity of BUB1 and BUBR1 staining varies only three- to fivefold between unattached and attached kinetochores. It is now evident that MAD2 localization is sensitive to microtubule attachments rather that kinetochore tension. When microtubule dynamics is suppressed by drugs (taxol or low concentrations of nocodazole or vinblastine) or low temperature, kinetochores are fully attached with microtubules, but no tension develops. As MAD2 is

 © 2004, Elsevier Inc. All Rights Reserved. 

not detected at these kinetochores, the conclusion was that MAD2 monitors microtubule attachment, but not the tension. These studies therefore reinforced an early notion that kinetochore tension is monitored by the checkpoint.

How tension is monitored by the checkpoint is not fully understood but it clearly involves aurora B/Ipl1, a protein kinase that is situated between sister kinetochores where tension is developed. Recent studies in yeast and mammalian cells showed that aurora B/Ipl1 is required for cells to arrest in mitosis when their kinetochores fail to establish tension despite being saturated with microtubules. Thus, aurora B was found to be essential for cells to arrest in the presence of taxol, a drug that suppresses microtubule dynamics and thus prevents kinetochores from developing tension. Despite this data, aurora-B/Ipl1 does not behave like the other checkpoint proteins, as it is not required for cells to arrest in mitosis in response to the loss of microtubule attachments. Aurora B/Ipl may therefore provide tension-sensing checkpoint functions.

AuroraB/Ipl1 has been proposed to be part of a mechanism that ensures that improperly attached kinetochores are provided the opportunity to establish proper connections to the spindle. The need for a self-correcting mechanism is evident because of the error-prone nature by which kinetochores establish microtubule connections. Merotelic and syntelic attachments are conditions where both kinetochores are connected to the same pole or when one kinetochore is attached to opposite poles, respectively. As kinetochores with merotelic and syntelic attachments are fully saturated with microtubules, a checkpoint that is only sensitive to microtubule occupancy at kinetochores will fail to detect these aberrant connections. AuroraB/Ipl is thought to monitor merotelic and syntelic attachments as these abnormal connections accumulated when this kinase was inactivated. Aurora B/Ipl1 is thought to resolve merotelic and syntelic attachments by stimulating the release of microtubules from these kinetochores and thus promoting new rounds of interactions. Given this scenario, it is possible that kinetochores lacking tension do not directly activate the checkpoint but do so as a result of microtubule detachments that is stimulated by aurora B kinase. This could explain why, in taxol arrested cells, there is on average one kinetochore that exhibits MAD2 staining. It may be possible that this is sufficient to arrest these cells in mitosis.

The mechanism by which aurora B promotes microtubule release from kinetochores has not been clarified but is likely mediated through MCAK, an unconventional kinesin-like protein that colocalizes with aurora B and functions to depolymerize microtubules.

### The Mitotic Checkpoint Monitors a Kinetochore Motor

CENP-E is a kinesin-like protein that binds to kinetochores at the onset of mitosis where it plays a critical role in establishing microtubule attachments. The link between CENP-E and the mitotic checkpoint was first established when cells whose kinetochores were depleted of CENP-E were found to arrest in mitosis. Cells defective for CENP-E functions characteristically accumulate a few chromosomes with monopolar attachments while the majority establish bipolar connections. The inability of monopolar chromosomes to establish bipolar connections is thought to reflect the importance of CENP-E in allowing kinetochores to capture the rare microtubules that emanate from the opposite pole. On the other hand, chromosomes that are positioned near the middle of the cell can establish bipolar attachments because the higher-frequency encounters with microtubules from both poles can compensate for the loss of CENP-E. Quantitative EM analysis showed that the bipolar attached kinetochores lack tension despite attaining near normal numbers of microtubule attachments. In contrast, the kinetochores of the monopolar chromosomes established very few microtubule connections. The defects of the monopolar versus the bipolar attached chromosomes are viewed differently by the mitotic checkpoint. Consistent with its role in detecting microtubule attachments, MAD2 accumulated at kinetochores of the monopolar chromosomes, while it was undetectable at kinetochores of the bipolar chromosomes. These findings clearly show that the mitotic delay induced by the loss of CENP-E may be due to loss of microtubule attachments and tension.

The precise role of CENP-E in the mitotic checkpoint has been somewhat controversial. Studies in human cells suggested that it was not essential for the checkpoint as cells were able to arrest in the absence of CENP-E. In contrast, *Xenopus* egg extracts depleted of CENP-E and hepatocytes derived from CENP-E null mice failed to delay mitosis in the presence of unaligned chromosomes. The difference in response however is most likely a function of whether the assembly of checkpoint proteins to kinetochores is dependent on CENP-E. In egg extracts, the localization of a number of checkpoint proteins to kinetochores was absolutely dependent on CENP-E. Thus, the failure of egg extracts to delay mitosis in the absence of CENP-E can be ascribed to the absence of checkpoint proteins at unattached kinetochores. This contrasts with human cells where checkpoint proteins present at kinetochores that were depleted of CENP-E. However, quantitative analysis showed that in mouse and human cells, CENP-E did affect the assembly of checkpoint proteins at kinetochores. In both species, kinetochores depleted of CENP-E had approximately two- to fourfold lower levels of

MAD1, MAD2, and BUBR1 than normal. However, this did not resolve why mouse cells failed to arrest in mitosis in the absence of CENP-E while human cells arrested. The discrepancy could be resolved if we take into account the fact that far fewer monopolar chromosomes accumulated in CENP-E depleted mouse cells than in human cells (two versus eight). Given that the reduction in the amount of checkpoint proteins at these kinetochores likely compromised their ability to generate the "wait anaphase" signal, the combined output from all of the unattached kinetochores in mouse cells may not reach a threshold level that is required to sustain a prolonged arrest. In human cells, this threshold may be achieved as a result of the higher numbers of unattached kinetochores. Indeed, both mouse and human cells depleted of CENP-E were able to arrest in mitosis if the number of unattached kinetochores were increased by using drugs that inhibit spindle formation.

The mechanism by which CENP-E function is monitored by the checkpoint is believed to be mediated by the BUBR1 kinase. This connection was uncovered when hBUBR1 was identified in a yeast two-hybrid screen for proteins that interacted with CENP-E. This interaction was subsequently validated when CENP-E and hBUBR1 were found to form a complex in cells. This finding coupled to the fact that hBUBR1 is an essential checkpoint protein suggested that hBUBR1 might act as a mechanosensor to monitor the activities of CENP-E at kinetochores (Figure 1). Consistent with this, hBUBR1 was found to be required for cells to arrest in mitosis when CENP-E functions were inhibited. The mechanism by which hBUBR1 monitors CENP-E activity remains to be clarified but the working hypothesis is that hBUBR1 kinase activity is sensitive to interactions between CENP-E and microtubules. In the absence of microtubule interactions, CENP-E assumes a conformation that stimulates hBUBR1 to generate the "wait anaphase" signal. When CENP-E is interacting productively with microtubules, hBUBR1 is then silenced. Recent *in vitro* data showed that both *Xenopus* and human CENP-E can directly bind to BUBR1 and this binding stimulated its kinase activity. As microtubules were not present in these reactions, it is possible that the interaction reflected the "checkpoint on" state. Addition of a CENP-E antibody to the CENP-E:BUBR1 complex inhibited kinase activity without disrupting the complex. The interpretation of this finding was that antibody binding altered the conformation of CENP-E so that it was unable to stimulate BUBR1 kinase activity. Thus, kinase activity of BUBR1 may be regulated allosterically by different conformational states of CENP-E. Although this model may indeed apply to the situation *in vivo*, the exact details of the mechanism remain to be clarified. For example, the *in vitro* dependence of BUBR1 kinase activity on CENP-E does not account for how cells that are depleted of CENP-E arrest in mitosis.

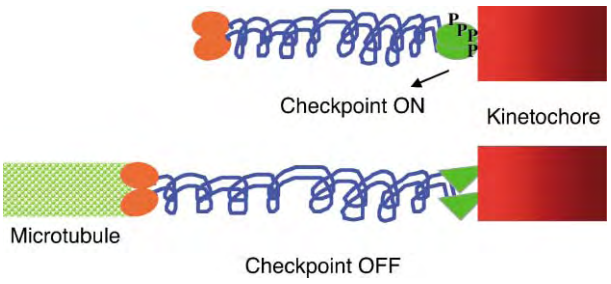



**FIGURE 1** BUBR1 kinase acts as a mechanosensor that monitors CENP-E at kinetochores. BUBR1 kinase (green) interacts near the carboxy-terminus of CENP-E where it is postulated to monitor the microtubule binding status of CENP-E. In the absence of microtubule attachments, BUBR1 is phosphorylated and activated (green oval) so that it generates the "wait anaphase" signal. When CENP-E is properly attached to microtubules, it undergoes a conformational change that inhibits BUBR1 kinase (green triangles) and thus extinguishes the "wait anaphase" signal.

## Inhibition of Mitotic Exit by the Checkpoint

Checkpoint proteins are functionally complex as they participate in multiple steps along the checkpoint signaling pathway. A confluence of genetic and biochemical studies showed that the target of the checkpoint is the APC, an E3 ubiquitin ligase that promotes the degradation of substrates that inhibit the onset of anaphase.

### Sequestration Model

Studies in budding and fission yeast identified checkpoint defective alleles of CDC20/Slp1, a protein that was shown biochemically to activate the APC by recruiting substrates to it. These CDC20 and Slp1 mutants were unable to bind to MAD2 and thus supported *in vitro* data that MAD2 can inhibit the ubiquitin ligase activity of CDC20-dependent APC. These findings coupled with the *in vivo* observation that MAD2 exhibited a rapid rate of turnover at unattached kinetochores led to a model that described how MAD2 might act as the diffusible wait anaphase signal. In this Sequestration Model, unattached kinetochores are thought to catalytically convert MAD2 into an "activated" state which upon release from kinetochores binds to CDC20 and sequesters it from the APC (Figure 2). The existence of an activated MAD2 initially gained support when it was discovered that recombinant MAD2 existed as monomers and oligomers (di- and tetramers), but the oligomeric form was more potent at inhibiting APC than the monomeric form *in vitro*. However, the existence of a

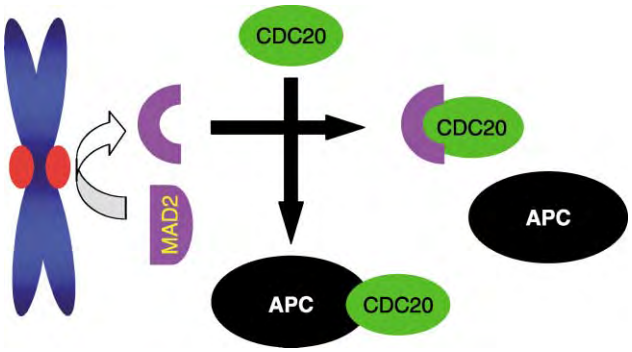



**FIGURE 2** Sequestration model. The MAD2 checkpoint protein (purple semicircle) transiently binds to unattached kinetochores (red ovals) where they are converted to a form that upon dissociation will bind to CDC20. CDC20 when associated with MAD2 is incapable of activating the APC. In this model, the pool of MAD2 that is released from kinetochores acts as the "wait anaphase" signal by indirectly preventing the activation of APC.

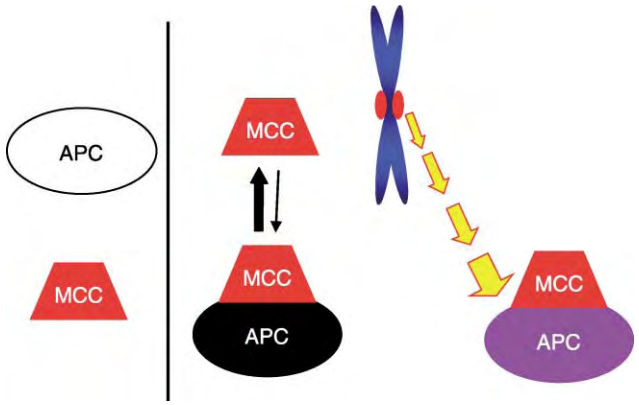



**FIGURE 3** Direct inhibition model. BUBR1, Bub3, CDC20, and MAD2 form the MCC that does not depend on kinetochores and exists throughout the cell cycle. The MCC exhibits little affinity for the interphase form of the APC (white oval). Upon entry into mitosis, the APC is rapidly activated by modifications (black oval) that if unchecked will prematurely drive cells out of mitosis. A preformed pool of MCC binds and inhibits the APC. The interaction between MCC and the mitotic APC is by necessity unstable. The role of unattached kinetochores (red ovals) is to generate a signal transduction cascade that targets and sensitizes the APC (purple) to prolonged inhibition by the MCC.

MAD2 oligomer *in vivo* has been elusive. Furthermore, MAD2 mutants that failed to form tetramers *in vitro* were found to be well tolerated in yeast. These data suggest that if an activated MAD2 does exist *in vivo*, it is unlikely to be a tetramer. Regardless, the biochemical fate of the MAD2 that is released from kinetochores is unknown. Thus, it remains open as to whether turnover of MAD2 at kinetochores is functionally significant for the checkpoint.

A similar model has been proposed for how BUBR1 inhibits the APC. As with MAD2, recombinant BUBR1 was found to also bind CDC20 *in vitro*. Not surprisingly, BUBR1 was found to block CDC20-dependent activation of the APC *in vitro*. This result led to the proposal that BUBR1 may act in parallel with MAD2 to inhibit the APC *in vivo*. The caveat of these studies was that the experimental design for the BUBR1 assays employed the interphase form of the APC, which is not the *in vivo* substrate for the mitotic checkpoint. The rationale for this approach was that the activity of the interphase APC was dependent on the addition of exogenous CDC20. This assay was limited in the sense that it was designed to identify factors that inhibited CDC20. The caveat of these studies was further highlighted when recombinant BUBR1 failed to inactivate mitotic APC, which should be the physiologically relevant substrate of the mitotic checkpoint.

## Direct Inhibitor Model

The Direct Inhibitor model differs from the sequestration model in that the APC is directly inhibited by checkpoint proteins and posits that unattached kinetochore sensitize the APC to its inhibitor (rather than generating a factor that sequesters an activator of the APC) (Figure 3). This model was proposed as a result of the discovery of the mitotic checkpoint complex (MCC), a factor that was identified in cell extracts that inhibited mitotic APC *in vitro*. The MCC was originally identified in Hela cells, where it was found to consist of near stoichiometric amounts of hBUBR1, hBUB3, CDC20, and MAD2. The interesting finding was that the MAD2 that was present in the MCC represented a very small fraction ($<5\%$) of the total pool of MAD2. Despite the presence of a large pool of monomeric MAD2, this pool was not found to inhibit the mitotic APC. Furthermore, MCC was more than 3000-fold more potent inhibitor of the APC than recombinant MAD2 (which was used in all the previous studies). The large difference in the inhibitory activities suggests that Hela cells do not express sufficient amounts of monomeric MAD2 to inhibit the APC. The mechanism by which MCC inhibited APC activity is not clear but is likely to depend on its ability to bind to the APC. When purified from cells, APC that was associated with MCC was found to be inactive, while APC that lacked MCC was highly active. Based on these findings, the MCC was proposed to be primary checkpoint inhibitor of the APC in mitotically arrested cells.

Although MCC was identified in mitotic Hela cells, it was subsequently found to be present and active throughout the cell cycle. Importantly, only APC that was isolated from mitotic cells was sensitive to inhibition by the MCC. How MCC distinguishes between interphase APC from mitotic APC is not

known but is most likely due to mitosis-specific modifications. Indeed, MCC appeared to preferentially bind to APC whose CDC27 subunit was mitotically phosphorylated. It is currently unknown if MCC directly interacts with CDC27 or through other APC subunits that undergo mitotic modifications. Regardless, the existence of MCC in interphase was unexpected, given that kinetochores, which are the sites thought to generate the inhibitor of the APC, are not fully formed until cells are in mitosis.

The importance of MCC in interphase becomes evident when one considers that the APC is rapidly activated at the onset of mitosis. A pre-formed pool of inhibitor provides the cell with a rapid way to inactivate APC. The inhibition of the APC by the MCC must be reversible so that cells can exit mitosis once the checkpoint is extinguished. Indeed, the interaction between MCC and the APC/C appears to be quite labile as APC activity in lysates prepared from mitotically arrested Hela cells cannot remain suppressed as ubiquitin ligase activity is reproducibly reactivated after a 10–15 min lag. This lag, however, could be extended if the extracts were supplemented with chromosomes (which provide the unattached kinetochores). Further studies using partially purified components showed that chromosomes did not enhance the inhibitory activity of MCC or the stimulating activity of CDC20, the likely target of kinetochores appears to be the APC/C.

The discovery of the MCC has led to a view that is fundamentally different from the predominant view that MAD2 cycles through kinetochores to bind and sequester CDC20 from the APC. In the "Direct Inhibitor model" the MCC acts independently of kinetochores to inhibit the APC. However, the interaction between MCC and APC is not stable unless unattached kinetochores are present. The role of unattached kinetochores is to sensitize the APC to prolonged inhibition by the MCC. The mechanism of APC sensitization is purely speculative. It is envisioned that unattached kinetochores initiate a kinase cascade that phosphorylates critical APC subunits that are required for MCC interactions. One expectation is that the modifications that sensitize APC to MCC inhibition must also be labile so that when the signaling cascade from kinetochores is extinguished, the APC will be de-sensitized. If unattached kinetochores generate a kinase cascade, it may target additional components besides the APC. One potential target is CDC20, as recent studies in *Xenopus* showed that phosphorylated CDC20 was unable to activate the APC.

The existence of the MCC has been confirmed in other species including *Xenopus* and yeast. Interestingly, MCC formation in budding yeast was also found to be independent of kinetochores. In the light of this finding, the molecular basis for the checkpoint-defective CDC20 mutants should be reinterpreted. The original interpretation was that these CDC20 mutants failed to be sequestered from the APC because they were unable to bind MAD2. From the perspective of the MCC, the checkpoint phenotype of these CDC20 mutants may result from their inability to assemble a functional MCC.

## Dual Roles for Mitotic Checkpoint Proteins

The discovery that the MCC contains proteins that are also localized at kinetochores suggested that they might play dual roles in the checkpoint. These proteins participate in monitoring kinetochore defects and then generating the "wait anaphase" signal. The same proteins can act independently of the kinetochore by being part of the MCC that directly inhibits the APC.

### Checkpoint Proteins can have Dual Functions

Until recently, it was technically not possible to design experiments that directly test if checkpoint proteins can serve dual functions. The solution came when it was discovered that the assembly of MAD2 onto kinetochores depended on hNuf2 and CENP-I. When kinetochores in Hela cells were depleted of hNuf2, they failed to assemble the checkpoint proteins, MPS1, MAD1 and MAD2. Similarly, kinetochores depleted of CENP-I failed to bind MAD1 and MAD2 (MPS1 was not investigated). In both cases, cells defective for hNuf2 or CENP-I functions failed to align chromosomes properly. Despite the lack of detectable MAD2 at kinetochores, the cells were able to delay mitotic exit. More surprisingly, this delay was still dependent on MAD2 as simultaneous depletion of MAD2 and hNuf2 by siRNA prevented accumulation of mitotic cells. Similarly, mitotic cells depleted of CENP-I rapidly exited mitosis when they were injected with MAD2 antibodies, while those injected with nonimmune antibodies remained in mitosis. These results are fully consistent with the idea that there are two functional pools of MAD2. The one that is present at kinetochores is involved in generating the "wait anaphase" signal, while the other pool acts independently of kinetochores. These studies indicate that the kinetochore-bound MAD2 is not essential. The characteristics of the kinetochore-independent pool of MAD2 are highly reminiscent of MCC, which is postulated to directly inhibit APC activity.

Aside from MAD2, there is also evidence to suggest that MPS1 and BUBR1 functions in a kinetochore-dependent and kinetochore-independent manner. MPS1 was one of the checkpoint proteins that was depleted

from kinetochores that lacked hNuf2. While hNuf2-depleted cells were shown to delay mitosis, cells directly lacking MPS1 failed to arrest in mitosis in response to microtubule poisons. The difference in outcomes is best explained if MPS1 possessed two distinct functions. As MPS1 is not associated with the MCC, its kinetochore-independent function remains uncertain.

Early studies of the human BUBR1 kinase showed that its kinase activity was essential for the mitotic checkpoint. Nevertheless, hBUBR1 purified from interphase cells as part of the MCC exhibits no detectable kinase activity, yet is fully capable of inhibiting APC. Likewise, *in vitro* studies showed that inhibition of the CDC20-dependent APC activity by recombinant BUBR1 can also be independent of its kinase activity. The apparent contradictory findings could be resolved by postulating that kinase activity of BUBR1 was required for the kinetochore while kinase activity was not required for its kinetochore-independent role as the MCC. This hypothesis has now been confirmed that were conducted in *Xenopus* egg extracts by BUBR1 depletion and add-back experiments. When extracts depleted of BUBR1 are reprogrammed with a kinase-dead mutant, they are no longer able to activate their checkpoint. Interestingly, if the BUBR1 (wild type or mutant) was supplemented to 20% of the endogenous level, it is sufficient to saturate all the kinetochores to levels seen in normal kinetochores. However, the presence of BUBR1 at kinetochores is still insufficient to activate the checkpoint unless the cytosolic pool of BUBR1 is also restored. This suggests that BUBR1 might provide two separable functions in the checkpoint. Whether the cytosolic BUBR1 acts by sequestering CDC20 or as part of the MCC is not clear. The finding that either wild-type or kinase-dead BUBR1 can restore the checkpoint to kinetochores that had assembled BUBR1 wild-type kinase but not the mutant supports the idea that its kinase activity was essential at kinetochores but not in the cytosol.

## "Wait Anaphase" Output Regulated by Checkpoint Proteins

Studies of hNuf2 and CENP-I led to the remarkable finding that MPS1, MAD1, and MAD2 do not appear to play an essential role at the kinetochore. How do kinetochores that lack MPS1, MAD2, and MAD2 maintain the checkpoint? This is most likely achieved in part by BUBR1 and BUB1 whose levels were not noticeably depleted. However, these proteins by themselves do not appear to be sufficient to generate a robust "wait anaphase" signal from unattached kinetochores. Cells defective for CENP-I accumulate a few monopolar chromosomes, while most chromosomes appear aligned (similar to loss of CENP-E). These cells are unable to sustain a prolonged mitotic arrest because the unattached kinetochores cannot generate sufficient amounts of "wait anaphase" signal to sustain a prolonged arrest. Consistent with this explanation, if the total number of unattached kinetochores was increased by disrupting the spindle with microtubule poisons, the collective output from a large number of unattached kinetochores must reach a critical threshold that is required for cells to arrest in mitosis. The caveat of this interpretation is that, after nocodazole treatment, kinetochores depleted of CENP-I exhibited detectable MAD2, although it was 20-fold lower than the level that is present at in-control cells. Nocodazole-treated Hela cells easily contain more than 20 unattached kinetochores (Karyotype $> 60$ chromosomes) and should therefore be able to generate a threshold level that can normally be achieved by a single unattached kinetochore.

## See Also the Following Articles

Cell Cycle: Control of Entry and Progression Through S Phase • Cell Cycle: DNA Damage Checkpoints • Chromosome Organization and Structure, Overview • Mitosis

## Glossary

**anaphase promoting complex (APC)** A multisubunit E3 ubiquitin ligase that promotes the proteolytic degradation of substrates such as cyclin B and securin in order for cells to exit mitosis.

**kinetochore** A macromolecular complex that is assembled at centromeres near the onset of mitosis that is essential for chromosomes to attach to the spindle.

**kinetochore tension** The force that is developed between sister kinetochores because of opposing poleward forces that attempt to separate them.

**monopolar and bipolar** States of attachment of chromosomes to either a single pole or to both poles of a spindle.

**"wait anaphase" signal** An inhibitory signal generated from unattached kinetochores that diffuses throughout the cell to block the onset of anaphase.

## Further Reading

Chan, G. K., Jablonski, S. A., Sudakin, V., Hittle, J. C., and Yen, T. J. (1999). Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome. *APCJ. Cell. Biol.* **146**, 941–954.

Fang, G., Yu, H., and Kirschner, M. W. (1998). Direct binding of CDC20 protein family members activates the anaphase- promoting complex in mitosis and G1. *Mol. Cell* **2**, 163–171.

Hauf, S., Cole, R. W., La Terra, S., Zimmer, C., Schnopp, G., Walter, R., Heckel, A., von Meel, J., Riedel, C. L., and Peters, J. M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol.* **161**, 281–294.

Hunter, A. W., Caplour, M., Coy, D. L., Honceck, W. O., Diez, S., Wordeman, L., and Howard, J. (2003). The kinesin-related protein MCAK is a microtubule depolymerase that forms an ATP-hydrolyzing complex at microtubule ends. *Mol. Cell* **11**, 445–457.

Liu, S. T., *et al.* (2003). Human CENP-I specifies localization of CENP-F, MAD1 and MAD2 to kinetochores and is essential for mitosis. *Nat. Cell Biol.* **5**, 341–345.

Mao, Y., Abrieu, A., and Cleveland, D. W. (2003). Activating and silencing the mitotic checkpoint through CENP-E-dependent activation/inactivation of BubR1. *Cell* **114**, 87–98.

Martin-Lluesma, S., Stucke, V. M., and Nigg, E. A. (2002). Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. *Science* **297**, 2267–2270.

McEwen, B. F., Chan, G. K. T., Zubrowski, B., Savoian, M. S., Sauer, M. T., and Yen, T. J. (2001). CENP-E is essential for reliable bioriented spindle attachment, but chromosome alignment can be achieved via redundant mechanisms in mammalian cells. *Mol. Biol. Cell.* **12**, 2776–2789.

Sudakin, V., Chan, G. K. T., and Yen, T. J. (2001). Checkpoint inhibition of the APC/C in Hela cells is mediated by a complex of BUBR1, BUB3, CDC20, MAD2. *J. Cell Biol.* **154**, 925–936.

Weaver, B. A., Bonday, Z. Q., Putkey, F. R., Kops, G. J. P. L., Silk, A. S., and Cleveland, D. W (2003). Centromere-associated protein-E is essential for the mammalian mitotic checkpoint to prevent aneuploidy due to single chromosome loss. *J. Cell Biol.* **162**, 551–563.

## Biography

Tim Yen is a senior member at the Fox Chase Cancer Center in Philadelphia, Pennsylvania. His major research interest is to characterize the molecular and biochemical composition of the kinetochore as a means to understand the mechanism of chromosome segregation in mammalian cells. He obtained his Ph.D. at the University of California at Santa Barbara and trained as a Postdoctoral Fellow at the Department of Biological Chemistry at the Johns Hopkins University School of Medicine. He was a Lucille Markey Scholar and a Scholar of the Leukemia and Lymphoma Society.

# Cell Death by Apoptosis and Necrosis

**Pierluigi Nicotera**
*MRC Toxicology Unit, Leicester University, Leicester, UK*

**Various genetically encoded programs involved in the signaling, initiation, and execution of cell death decide cells' fate during development and adult life. These programs can execute physiological cell death during development or tissue turnover, but are also involved in the inappropriate elimination of cells under pathological conditions. Because balanced cell turnover is essential for life, defects in cell elimination can also result in disease, the foremost example being cancer. In many circumstances, both physiological cell death and cell death in pathological settings have similar morphological and biochemical characteristics. Perhaps the best characterized biochemical and morphological changes during a cell death program are those defined as apoptosis. Apoptosis is characterized by condensation and fragmentation of the nucleus with shrinkage of the cytoplasm and exposure of surface molecules that facilitate recognition of the dying cells by phagocytes. However, other types of cell death are present and are strictly regulated *in vivo*, including cell lysis/necrosis or autophagy. Imbalance in cellular calcium regulation has been involved in both apoptotic and non-apoptotic cell death. Calcium can be a signal for cell death or simply a downstream consequence of the activation of the death machinery.**

## $Ca^{2+}$ as a Signal for Cell Death

A sustained $Ca^{2+}$ overload, such as that resulting from dysfunction of the main routes of $Ca^{2+}$ entry or efflux or from the irreversible loss of intracellular buffering capacity, can be lethal. The idea that $Ca^{2+}$ may be cytotoxic dates back to A. Fleckenstein's suggestion in 1968 that excessive entry of $Ca^{2+}$ into myocytes could be the underlying mechanism of cardiac pathology following ischemia. Subsequent studies showed that agonist stimulation or cytotoxic agents could cause lethal $Ca^{2+}$ entry into cells. Cellular $Ca^{2+}$ overload involves multiple intra- and extracellular routes, most of which are also used for physiological signaling, which implies that not only alterations of the normal $Ca^{2+}$ homeostasis but also changes in $Ca^{2+}$ signaling can have adverse effects. The following have been shown in a large number of experimental paradigms: (1) Direct sustained elevation of $[Ca^{2+}]_i$ (e.g., by exposure of cells to ionophores or to conditions that cause prolonged gating of inward-directed channels) causes cell death. (2) A $[Ca^{2+}]_i$ elevation precedes cell death induced by pathophysiological stimuli. (3) Prevention of $[Ca^{2+}]_i$ elevation during such experiments can inhibit cell death. (4) Alterations of $Ca^{2+}$-signaling pathways (e.g., potentiation or inhibition of $Ca^{2+}$ currents) can result in cytotoxicity.

## Executors of $Ca^{2+}$ Death Signals

Intracellular $Ca^{2+}$ signals can set off cell demise via $Ca^{2+}$-dependent processes, change the mode of cell death from apoptosis to necrosis, or synergize with elements of the apoptotic death program. In particular, $Ca^{2+}$-activated proteases (calpains) can synergize with caspases and amplify apoptotic death routines, while modulators of apoptosis such as members of the Bcl-2 protein family can modulate $Ca^{2+}$ compartmentalization. Hydrolytic enzymes, which include calpains, various DNAses, and lipases, are the best-characterized effectors of cell death directly mediated by calcium overload. Calpains are $Ca^{2+}$-activated cysteine proteases that have been implicated in toxic cell death in the liver and in excitotoxic neuronal death. Calcium-dependent DNAses can be responsible for DNA degradation, although the nature of the $Ca^{2+}$-dependent enzyme(s) responsible for the typical oligonucleosomal DNA cleavage has remained elusive. Among lipases, the $Ca^{2+}$-dependent phospholipase $A_2$ ($PLA_2$) has been implicated in neurotoxicity. Its activation results in the release of arachidonic acid and related polyunsaturated fatty acids, which are further metabolized by lipoxygenases or cyclooxygenases with concomitant generation of reactive oxygen species (ROS). In addition, $PLA_2$ activation generates lysophosphatids that alter the membrane structure, which may facilitate $Ca^{2+}$ influx and $Ca^{2+}$ release from internal stores.

 © 2004, Elsevier Inc. All Rights Reserved. 

## $Ca^{2+}$, Excitotoxicity, and Death During Brain Ischemia

Excitotoxicity is a phenomenon typically encountered in neurons or myocytes following receptor stimulation by excitatory amino acids that exceeds the physiological range with respect to duration or intensity. Typical excitotoxic stimulators are capsaicin, acetylcholine, or – most important in the central nervous system – glutamate. Direct injection of glutamate is selectively neurotoxic *in vivo*. Also, inhibition of excess synaptic activity by inhibitors of glutamate receptor subtypes (mainly N-methyl-D-aspartate, or NMDA) protects neurons from hypoxia. Generally, excitotoxicity is induced by conditions favoring glutamate accumulation in the extracellular space. Typical conditions leading to increased extracellular glutamate concentration are depolarization of neurons, energy depletion due to hypoglycemia or hypoxia, or defects in the glutamate reuptake systems.

Overall, three different lines of evidence suggest the key role of $Ca^{2+}$ in excitotoxicity: (1) There is an obvious increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in both *in vivo* and *in vitro* models of excitotoxic cell death. This has been observed in ischemic brain and in brain slices exposed to NMDA agonists or anoxia. In addition, glutamate-stimulated $Ca^{2+}$ influx in cultured neurons has been shown by the $Ca^{45}$ technique, and increased $[Ca^{2+}]_i$ after NMDA stimulation has been observed repeatedly using fluorescent probes. (2) Prevention of $Ca^{2+}$ entry into the cell by removal of extracellular $Ca^{2+}$ depletion of NMDA or by pharmacological inhibition of glutamate receptors or voltage-dependent $Ca^{2+}$ channels prevents neuronal death in many paradigms of excitotoxicity. (3) Prevention of neurotoxicity by inhibition of downstream effects of $Ca^{2+}$ overload strongly suggests a causal role of $Ca^{2+}$ in excitotoxicity. Intracellular $Ca^{2+}$ chelators can prevent ischemic damage *in vivo* and excitotoxic neuronal damage *in vitro*. Also, inhibition of effectors of $Ca^{2+}$ toxicity such as calmodulin, calcineurin, and bNOS protects neurons from the toxicity of excitatory amino acids. On the other hand, there could potentially be other routes for $Ca^{2+}$ entry under excitotoxic conditions.

Using a model of hypoxia (oxygen/glucose deprivation, or OGD), a new lethal pathway that involves the activation of a cation conductance ($I_{OGD}$) has recently been unveiled. This leads to neuronal $Ca^{2+}$ overload in the absence of excitotoxic stimulation. $I_{OGD}$ requires the TRMP7 ion channel protein, a member of the TRP (transient receptor potential) cation channel super family. Gating of TRMP7 occurs because of the generation of an excess of reactive oxygen/nitrogen species in anoxic neurons. Because gating TRMP7 allows $Ca^{2+}$ entry into the neuron and $Ca^{2+}$ can stimulate further radical production, a vicious loop leading to sustained $Ca^{2+}$ overload is established in the absence of NMDA-R stimulation by excess glutamate.

In addition to excessive entry of $Ca^{2+}$ trough membrane channels, mitochondrial $Ca^{2+}$ sequestration and subsequent release play a central role in ischemia- or glutamate-mediated cell death. Nevertheless, mitochondrial $Ca^{2+}$ release and increased $Ca^{2+}$ influx into neurons cannot fully account for the irreversible build-up of intracellular $Ca^{2+}$ after excitotoxic stimulation. The bulk increase in cellular $Ca^{2+}$ should be rectified over time, unless cellular $Ca^{2+}$ extrusion is inhibited. Inhibition of cellular $Ca^{2+}$ efflux from cells is sufficient to trigger cell death in non-neuronal cells, and in neurons may be brought about by oxidative damage downstream of mitochondrial dysfunction. Recent work shows that calpains can cleave the plasma membrane $Na^+/Ca^{2+}$ exchanger (NCX) in brain ischemia and in cerebellar granule neurons exposed to glutamate. Calpain-mediated NCX proteolysis is necessary for the delayed excitotoxic $Ca^{2+}$ deregulation leading to neuronal death (for a schematic summary of excitotoxicity, see Figure 1).

## Apoptosis, Necrosis, and Other $Ca^{2+}$-Dependent Forms of Cell Death in Brain Ischemia

Neuronal demise and neurological dysfunction in brain ischemia are clearly due to several components. Apoptosis and necrosis, in their classical definition, are two fundamentally different modes of cell death. Whereas apoptosis is characterized by a preservation of membrane integrity until the cell is phagocytosed, this is not the case in necrosis/lysis of cells. The duration and extent of $Ca^{2+}$ influx may determine if neurons survive, die by apoptosis, or undergo necrotic lysis. Very low $[Ca^{2+}]_i$ or the prolonged inhibition of $Ca^{2+}$ influx may be neurotoxic. A continuous moderate increase in $[Ca^{2+}]_i$, such as that produced by a sustained slow influx, may cause apoptosis, whereas an exceedingly high influx causes rapid cell lysis. For example, stimulation of cortical neurons with high concentrations of NMDA results in necrosis, whereas exposure to low concentrations causes apoptosis. Accordingly, neuronal death in experimental stroke models is predominantly necrotic in the ischemic core, but apoptosis occurs in the less severely compromised penumbra or border regions. The same applies to several other neuropathological conditions in which apoptosis and necrosis have been observed to occur simultaneously. One sensor that switches neurons toward one of the two fates is the ability of cells to generate ATP. A complete de-energization of the cell (e.g., failure of all mitochondria and of glycolysis) does not allow the

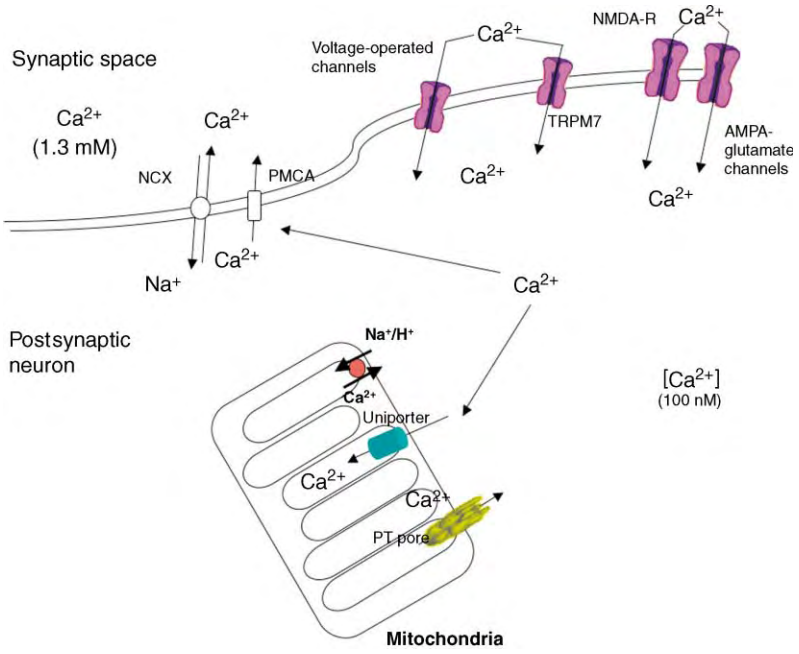



**FIGURE 1** The term neuronal excitotoxicity defines as consequences of excessive stimulation of postsynaptic glutamate receptors. Excess glutamate in the synapse causes gating of AMPA and NMDA receptors. The former allows entry of $Na^+$ and $Ca^{2+}$ into the neuron. $Na^+$ hyperpolarizes the plasma membrane and removes the $Mg^{2+}$ block from the NMDA receptor (the NMDA receptor is normally "plugged" by $Mg^{2+}$). The NMDA receptor is then gated to $Ca^{2+}$ that flows into the neuron. Additional routes for $Ca^{2+}$ entry involve the TRPM7 cation channel and voltage-operated $Ca^{2+}$ channels. $Ca^{2+}$ accumulates in mitochondria as amorphous calcium phosphate. When the buffering capacity of mitochondria is overwhelmed, $Ca^{2+}$ is released in a process known as permeability transition (PT). Efflux of $Ca^{2+}$ from the neuron is operated by the NCX and the PMCA. When mitochondria release their $Ca^{2+}$ and the efflux systems are unable to extrude the excess intracellular $Ca^{2+}$, $Ca^{2+}$ overload becomes irreversible and causes neuronal demise.

ordered sequence of changes required for the apoptotic demise. In such a case, other processes result in rapid, uncontrolled cell lysis/necrosis. Therefore, under conditions of $Ca^{2+}$ overload, apoptosis ensues when sufficient energy production (ATP) is available to execute the death program. The main ATP-requiring step for the execution of apoptosis is the formation of the apoptosome protein complex between cytochrome c released from damaged mitochondria, the cytosolic protein APAF-1, and procaspases. Either ATP or dATP is required to promote the functional activation and assembly of this complex, which then leads to caspase activation and cell breakdown.

## $Ca^{2+}$ Signals as Subroutines of the Apoptotic Program

The role of $Ca^{2+}$ in apoptosis signaling was initially suggested by studies in thymocytes and lymphocytes showing that sustained $Ca^{2+}$ increases could trigger the DNA fragmentation typical of apoptosis and all the other features of the apoptotic demise. $Ca^{2+}$ may be central in the unfolding of the apoptotic program at different stages. $Ca^{2+}$-mediated mitochondrial permeability transition is recognized as one important mechanism for cytochrome c release and caspase activation. In addition, ER-mitochondrial $Ca^{2+}$ fluxes are modulated by the Bcl-2 protein family, and caspase-mediated cleavage of $Ca^{2+}$ transporters then results in further disruption of ER $Ca^{2+}$ handling, with subsequent mitochondrial $Ca^{2+}$ overload and permeability transition. Ample evidence has documented crosstalk among calpains, caspases, and other protease families, which alters the downstream effects of these families on cellular $Ca^{2+}$ fluxes. Finally, $Ca^{2+}$-regulated processes are also involved in the ultimate fate of dying cells, their clearing by phagocytes due to $Ca^{2+}$-dependent exposure of surface recognition molecules and secondary lysis. The latter can be brought about by caspase-dependent cleavage of the plasma membrane $Ca^{2+}$ ATPase (PMCA), which results in a secondary $Ca^{2+}$ overload and the activation of $Ca^{2+}$-dependent mechanisms causing cell lysis.

### See Also the Following Articles

Autophagy in Fungi and Mammals • Bax and Bcl2 Cell Death Enhancers and Inhibitors • Calpain • Caspases and Cell Death • Plasma-Membrane Calcium Pump: Structure and Function

## Glossary

**calpains** A family of $Ca^{2+}$-activated proteases that mediates some of the physiological effects of $Ca^{2+}$ signals but that can also mediate cell death. Substrates include cytoskeletal proteins, other proteases, and $Ca^{2+}$ transport proteins.

**caspases** Cysteine aspartases that are activated during inflammatory processes and in apoptosis.

**NCX** The sodium–calcium exchanger that operates the transport of these two ions depending on their electrochemical gradients.

**plasma membrane $Ca^{2+}$ pump ATPase (PMCA)** Transmembrane $Ca^{2+}$ pump that uses ATP to pump $Ca^{2+}$ out of cells.

## Further Reading

Aarts, M., Iihara, K., Wei, W.-L., Xiong, Z.-G., Arundine, M., Cerwinski, W., MacDonald, J. F., and Tymianski, M. (2003). A key role for TRPM7 channels in anoxic neuronal death. *Cell* **115**, 863–877.

Ankarcrona, M. D. J., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., and Nicotera, P. (1995). Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **14**, 961–973.

Han, B. H., Xu, D., Choi, J., Han, Y., Xanthoudakis, S., Roy, S., Tam, J., Vaillancourt, J., Colucci, J., Siman, R., *et al.* (2002). Selective, reversible caspase-3 inhibitor is neuroprotective and reveals distinct pathways of cell death after neonatal hypoxic-ischemic brain injury. *J. Biol. Chem.* **277**, 30128–30136.

Lee, J. M., Zipfel, G. J., and Choi, D. W. (1999). The changing landscape of ischaemic brain injury mechanisms. *Nature* **399**, A7–14.

Lipton, S. A. (1996). Similarity of neuronal cell injury and death in AIDS dementia and focal cerebral ischemia: Potential treatment with NMDA open-channel blockers and nitric oxide-related species. *Brain Pathol.* **6**, 507–517.

Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003). Regulation of cell death: The calcium apoptosis link. *Nat. Rev. Mol. Cell. Biol.* **4**, 552–565.

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003). BAX and BAK regulation of endoplasmic reticulum $Ca^{2+}$: A control point for apoptosis. *Science* **300**, 135–139.

## Biography

Pierluigi Nicotera is Professor of Neuroscience and Professor of Toxicology at the University of Leicester, where he is also the Director of the UK Medical Research Council Toxicology Unit. His main research interest is in the mechanisms of cell injury and death in disease and toxic conditions. His research has contributed to the understanding of the mechanisms in which $Ca^{2+}$ can cause cell dysfunction and death in neurons and non-neuronal cells.

# Cell Migration

**J. Victor Small and Emmanuel Vignal**
*Austrian Academy of Sciences, Salzburg, Austria*

**The morphogenesis of multicellular organisms involves the extensive migration of cells from primordial sites to locations destined for specific tissue development. Among the most dramatic are the movements of cells of the neural crest, which travel from the neural tube to distant sites where they differentiate into diverse cell lineages. The fusion of epithelial layers, such as those which occur during closure of the neural tube, entails coordinated cell migration. In the adult, wound closure and tissue repair depends on the migration of surrounding cells to effect the regeneration process. In defense against foreign organisms, cells of the immune system are mobilized and migrate to the sites of inflammation to engage with the enemy. In another context, cell migration contributes to the dissemination of malignant cells in the spread of cancer. Understanding cell migration has therefore much to do with life and death, which concerns all.**

**Metazoan cells migrate by a crawling mechanism that entails continuous changes in shape. Crawling, in turn, requires traction and this is provided by the development of transient points of anchorage with the connective tissue scaffold and with other cells. In both processes, shape change and traction, the intracellular polymer framework of the cell, the so-called cytoskeleton, plays a central role. Changes in the cytoskeleton framework are influenced, in turn, by the chemical and mechanical properties of the surrounding matrix, so there is an active crosstalk between the two. Most of what is known about the mechanisms of cell migration comes from studies of cells moving on planar surfaces *in vitro* and from investigations of the test tube properties of cytoskeleton polymers and their associated proteins. However, parallel studies indicate that the basic principles derived from these approaches apply also to cell movement in a tissue environment.**

## The Cytoskeleton

The cytoskeleton is composed of three distinct, but interlinked networks of polymers, composed of actin, tubulin, and proteins of the intermediate filament family, together with many associated proteins. The term cytoskeleton is a misnomer, because the cytoplasmic filament networks that make it up are in a state of continuous turnover and rearrangement. Cell migration is driven primarily by the programmed turnover of the actin cytoskeleton, but microtubules exert an important influence on polarization and guidance, which is required for directional motility. Intermediate filaments do not appear to play an important role in cell motility, but a subtle modulatory role cannot yet be excluded.

### The Actin Cytoskeleton

The actin cytoskeleton is composed of actin filaments organized in networks and bundles: the salient features of the actin cytoskeleton in a fibroblast are illustrated in Figures 1 and 2. The cell periphery is delimited by either bundles of actin filaments, running parallel to the cell edge, or by dense actin meshworks (Figure 2A) that are commonly punctuated by small, radial actin bundles, termed microspikes or filopodia (Figure 2B). The actin meshworks (Figure 2) are the structural component of the sheet-like lamella regions at the cell periphery called lamellipodia (or ruffles, when they fold upwards. Figure 3). The body of the cell is pervaded by a loose network of actin filaments, a proportion of which are organized into prominent bundles, called stress fibers. According to cell type, the ratio of peripheral actin meshworks to stress fibers differs and as a general rule, cells that migrate faster have fewer stress fiber bundles in the body of the cell. The different organizations of actin filaments that make up the subcompartments of the actin cytoskeleton are signaled via pathways involving different members of the Rho family of small GTPases: Rho for stress fibers, Rac for lamellipodia, and Cdc42 for filopodia.

One important feature of the actin cytoskeleton is its linkage to the cell membrane, which explains its pivotal influence on cell form. Major sites of linkage occur at the termini of the stress fiber bundles, which in turn correspond to sites of adhesion of the cell to the extracellular matrix. Because of their focal nature, these sites are called focal adhesions (Figure 2C). They comprise more than 50 structural, adaptor, and signaling proteins that form and regulate the linkage of the actin cytoskeleton, via transmembrane receptors of the integrin family, to the extracellular matrix. The precursors of these anchorage sites are assembled in

 © 2004, Elsevier Inc. All Rights Reserved. 

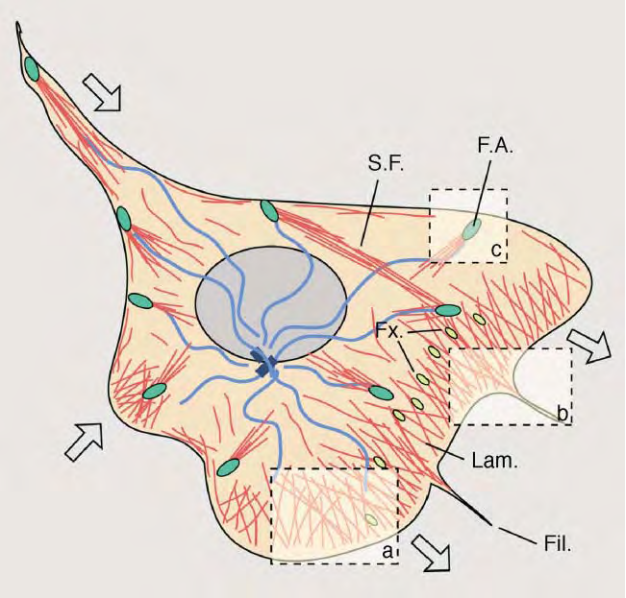



**FIGURE 1** General features of the actin cytoskeleton of a migrating cell. Actin filaments are in red. Protrusion of the cell is driven by the formation of sheet-like meshworks (lamellipodia, Lam) and rod-like projections (filopodia, Fil). Early adhesions form beneath protrusions as focal complexes (Fx) and can mature into larger, focal adhesions (FA) at the termini of actin bundles, called stress fibers (SF). The polarization of the cell is reflected in a polarization of the adhesion pattern, with stationary focal complexes and focal adhesions at the front and sliding focal adhesions at the rear. Microtubules (blue) interact with adhesions and influence their turnover. Details of the boxed areas are provided in Figure 2.

association with lamellipodia and filopodia at the cell periphery and are called focal complexes (Figure 1).

## Phases of Movement

In general terms, the mode of translocation of a cell can be divided into three phases: (1) protrusion of a cell front; (2) the development of adhesion to the substrate for the purpose of traction; and (3) retraction of the cell rear.

### Protrusion

In the first step of movement, the cell protrudes lamellipodia and filopodia: sheet-like and rod-like processes $\sim$0.2 μm thick and several microns in length (Figures 3 and 4). The rate of protrusion varies from $\sim$2–15 μm $min^{-1}$. Protrusion is based on the unidirectional polymerization of actin, whereby actin monomers are inserted at the tips of actin filaments where they abut the cell membrane at the leading front (Figures 2A and 2B). Complexes of proteins are recruited at these membrane sites that signal and drive actin polymerization, downstream from Rac and Cdc42. Additional proteins are responsible for cross-linking the formed actin filaments into meshworks (lamellipodia) and bundles (filopodia) and for disassembling actin filaments at the base of lamellipodia and filopodia to provide building blocks for further protrusion. Protrusion therefore involves a regulated "treadmilling" of actin monomers from the front to the rear of lamellipodia and filopodia. This produces a "retrograde flow" of actin which is accompanied by a retrograde flow of associated material in protruding zones. The mechanisms underlying the delivery of actin and other components to the front of lamellipodia, to support polymerization and retrograde flow have yet to be clarified, but likely involve the engagement of myosin motor molecules. In their role as protruding organelles, lamellipodia and filopodia are major sites of actin filament generation in a motile cell and contribute filaments also to the cytoskeleton network that spans the cell.

### Adhesion

In addition to their role in protrusion, lamellipodia and filopodia initiate adhesion to the extracellular matrix. This involves the recognition of matrix ligands on the outside and the accumulation of integrins and proteins of the adhesion machinery to form specific focal points, the focal complexes (Figure 1). The accumulation of proteins in focal complexes is likely to be linked to the cycling of proteins through lamellipodia and filopodia by retrograde flow.

Focal complexes do not move relative to the substrate and can experience one of two fates. They either exist transitorily for a few minutes and disperse, or they enlarge and differentiate into larger anchorage sites at the ends of stress fiber bundles, the focal adhesions. The transition from focal complexes to focal adhesions is linked to a switch in signaling from Rac/Cdc42 to Rho and to the engagement of muscle-type myosin with actin to form contractile bundles.

The regulation of adhesion formation and turnover is a complex process that involves both enzymatic and mechano-sensory pathways. Both focal complexes and focal adhesions are enriched in tyrosine kinases as well as their substrates and changes in their activities modulate adhesion complex turnover. Focal adhesion formation and maintenance depends also on mechanical stress in the actin cytoskeleton. This is illustrated dramatically by their disappearance when cells are treated with drugs that inhibit the interaction between muscle type myosin and actin. Mechano-sensory mechanisms therefore play a role in regulating adhesion dynamics, most likely through mechanically induced

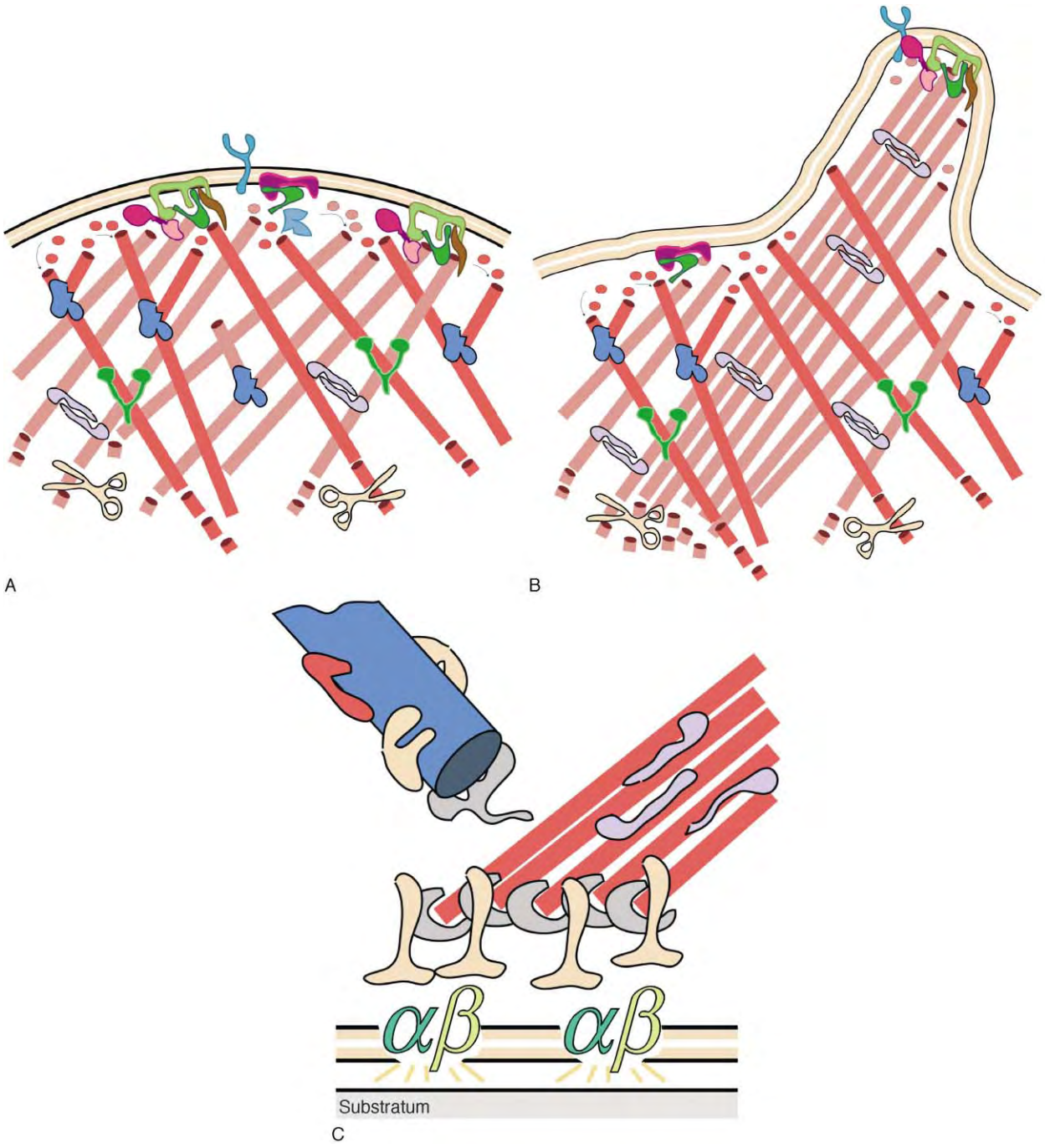



**FIGURE 2** Schematic details of the boxed areas in Figure 1. Lamellipodia (A) are formed by the generation of an actin meshwork through the initiation of actin polymerization and the addition of actin monomers (red dots) at the cell membrane. Protein complexes recruited to the membrane control the polymerization process in response to signaling stimuli. Other actin-binding proteins serve to cross-link the actin network. Turnover of the lamellipodium components occurs through the depolymerization of most of the actin filaments towards the base of the lamelipodium by destabilizing and severing factors (scissors). Filopodia (B) are formed by the bundling of lamellipodia filaments, by additional proteins (antiparallel bars), followed by extension via actin polymerization at the tip. Focal adhesions (C) are sites of linkage of actin filament stress fiber bundles to the extracellular matrix via transmembrane matrix receptors called integrins ($\alpha\beta$). A complex of at least 50 structural, adaptor, and signaling proteins are recruited to these sites. The actin filaments are bundled by the cooperation of myosin and actin cross-linking proteins. Microtubules (blue cylinder) polymerize into focal adhesions and impart signals via associated proteins, that promote adhesion turnover.

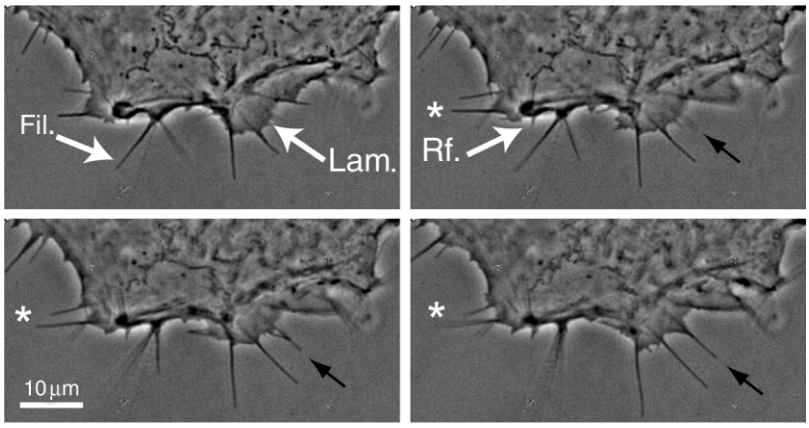



**FIGURE 3** Lamellipodia (Lam.) and filopodia (Fil.) in a living fibroblast. Figure shows a sequence of video frames (20 s apart) of the periphery of a living goldfish fibroblast seen in phase contrast optics. These cells show a high incidence of filopodia. The white asterisk indicates a filopodium situated close to the ruffling region that lifted upwards during the sequence. The black arrow indicates the position of a protruding filopodium. These images were kindly supplied by Dr. Irina Kaverina.

conformational changes of specific proteins in adhesion sites.

### Adhesion Asymmetry and Polarization

For a cell to move it must develop an advancing front and a retracting tail (Figure 4). This polarization process is reflected in an asymmetry of the pattern of adhesions developed with the extracellular matrix. It is interesting to consider how this asymmetry is established as it is relevant to the question of how polarization is determined.

As long as a cell edge protrudes, focal complexes are created in newly won territory under lamellipodia and filopodia and a proportion differentiates into focal adhesions. In general, however, lamellipodia and filopodia do not protrude in a persistent manner. Instead, forward advancement is the net result of repetitive protrusion and retraction events. Retraction involves the withdrawal of lamellipodia or filopodia, or their backfolding, as ruffles. When this occurs, focal complexes dissolve, or convert into focal adhesions that tether the retracted cell edge.

In the above context, polarization can be considered the result of regional changes in the ratio of protrusion and retraction events at the cell edge. Thus, at the advancing front of a migrating cell the duration of protrusion exceeds that of retraction. Elsewhere, at the rear and flanks, protrusion can occur, but retraction dominates. In the simplest terms, the front edge of a migrating cell is accordingly populated by focal complexes and the rear edge by focal adhesions. In many cases, focal adhesions are also formed behind the front edge and contribute to traction. This asymmetry of adhesion site development is the hallmark of a polarized cell.

### Traction and Retraction

An important difference between focal adhesions at the front and rear of migrating cells is that those at the front remain fixed relative to the substrate, whereas those at the retracting rear and flanks can slide. Studies of fibroblasts moving on flexible substrates show that the anterior focal adhesions exert more stress per unit area on the substrate than those at the rear. The anterior adhesions therefore provide anchorage points for the actin cytoskeleton that support the retraction of the trailing cell body. Retraction itself is driven by the interaction of myosin with the actin filaments of the cytoplasmic network.

## Microtubules and Cell Guidance

When fibroblasts are treated with drugs that dissassemble microtubules they become depolarized and extend protrusions in all directions. At the same time, tension in the actin cytoskeleton increases and focal adhesions grow in size. These changes are linked to an increase in the activity of Rho. Microtubules are therefore required for polarization and they exert their influence on polarity via a cross-talk with the actin cytoskeleton. The cross-talk takes place between the tips of microtubules and the focal adhesion sites at the ends of actin bundles (Figures 1 and 2C).

Microtubules grow from the centrosome towards the cell periphery, but not in a continuous manner. Instead, they exhibit alternating periods of polymerization and depolymerization in a mode referred to as

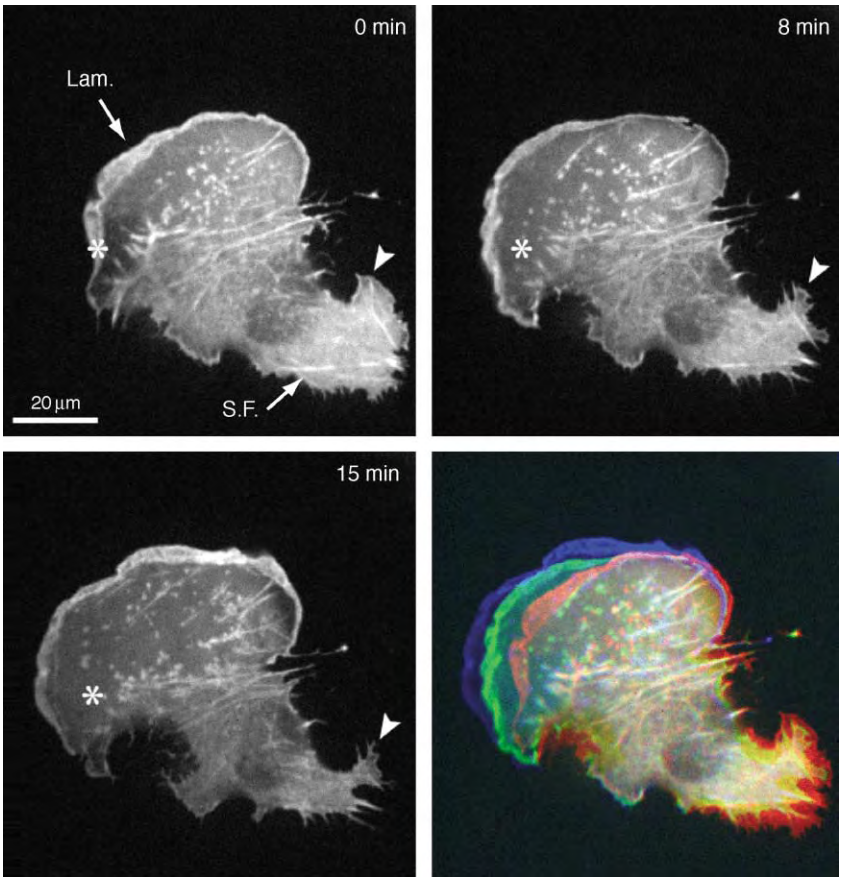



**FIGURE 4** A motile melanoma cell, showing a protruding front and a retracting rear. The cell was transfected with GFP-actin and images were recorded by fluorescence optics. The protruding front is marked by a prominent lamellipodium (Lam.). When moving on laminin these cells show few filopodia. The extent of protrusion and retraction during the video sequence is indicated by reference to the fixed points marked by an asterisk and the arrowhead as well as by the color overlay of the frames. Retraction is mediated by the shortening of actin stress fiber bundles that connect to the cell rear (e.g., S.F.).

"dynamic instability." When they reach the cell periphery, microtubules target focal adhesions and this targeting can occur in a repetitive manner. Targeting is more frequent at retracting cell edges and is correlated with the dispersal of focal adhesions or with their release from the substrate. Microtubules bind various molecules including Rho family exchange factors that have an influence on the turnover of the actin cytoskeleton. Some of these associate with proteins concentrated at the growing tips of microtubules. Microtubules then serve as transmission elements to target potential regulators of actin cytoskeleton turnover to focal adhesions. Transmission most likely involves the engagement of microtubule motors to deliver the regulators to the microtubule tip.

## Microtubules and Contractility

The global effect of microtubules is to reduce cell contractility. At the level of focal adhesions, microtubules appear to mediate the localized relaxation of actomyosin interactions at the ends of stress fibers, to promote focal adhesion disassembly. This occurs in a feedback mode, whereby an increase in mechanical stress at adhesion sites signals the polymerization of microtubules into them. The influence of microtubules on cell polarity can be explained by their modulation of focal adhesion turnover, in a spatially defined manner involving a mechano-sensory feedback on microtubule dynamics.

The dependence of a cell on microtubules to maintain polarity is linked to the extent of formation of focal adhesions. Very motile cells, such as neutrophils do not form stress fibers and typical focal adhesions and are less dependent on microtubules for directional locomotion. Likewise, fish epidermal keratocytes exhibit focal complexes, but lack focal adhesions and can also migrate without microtubules. Some cells appear to have the inherent ability to segregate protruding and contractile domains. But most cells are unable to maintain this segregation without the modulatory input of microtubules.

## Migration *in vivo*

Cells migrating in tissues exhibit both filopodia and lamellipodia. These thin protrusive extensions are ideal for exploring and penetrating tissue spaces. They are also well suited for intercalating between cells, such as during the migration of leukocytes across endothelial layers. Stress fibers, as seen in cultured cells, are not obvious features of cells moving in a three-dimensional environment. However, inhibition of the Rho pathway – which signals contractility – inhibits the migration of primordial cells during embryogenesis and prevents tail retraction during the transendothelial migration of monocytes. Migration *in vivo* therefore involves protrusion of lamellipodia and filopodia signaled by Rac and Cdc42 as well as retraction signaled by Rho. The closure of epithelial layers during embryogenesis is likewise dependent on protrusion and retraction events, whereby the contractility of actin bundles parallel to the epithelial boundary contributes to closure in a zipper-like mode.

Further factors important for cell migration have not been discussed here. These include the cues initiating migration at an appropriate timepoint and the gradients of chemotactic factors that define the destinations of migrating cells. Needless to say, these must impinge on the network of pathways that signal to the locomotion machinery.

## See Also the Following Articles

Actin Assembly/Disassembly • Centromeres • Centrosomes and Microtubule Nucleation • Focal Adhesions • Integrin Signaling

## Glossary

**actin filament** A filament of ~8 nm in diameter, made up from two helical strands of actin monomers.

**filopodia** Bundles of unipolar actin filaments, ~0.2 μm in diameter, that protrude from the cell edge, normally in association with lamellipodia and ruffles. Filopodia and lamellipodia are interconvertible assemblies of actin filaments.

**focal adhesions** Longer-lived adhesion foci that link to contractile actin bundles (stress fibers) in the actin cytoskeleton. Both focal complexes and focal adhesions harbor more than 50 proteins, involved in structural and signaling activities.

**focal complex** A site of early adhesion to the extracellular matrix, formed beneath a lamellipodium or a filopodium. Focal complexes can either form and dissolve, within 1–2 mins, or they can mature into focal adhesions.

**integrins** A family of transmembrane receptor molecules that cluster at adhesion foci to link the matrix on the outside to the actin cytoskeleton on the inside.

**lamellipodia** Thin, membrane-bound leaflets of cytoplasm, 0.2–0.3 μm thick and up to several microns wide that are protruded at the cell edge, close to and parallel to the substrate. They are composed of unipolar networks of actin filaments.

**Rho (Ras homology) proteins** A subfamily of small GTPases whose roles include signaling to the actin cytoskeleton.

**ruffles** Manifestations of lamellipodia, protruding upward from the dorsal cell surface, and migrating generally rearwards over it. Lamellipodia can also fold upwards and rearwards to form ruffles.

## Further Reading

Bray, D. (2000). *Cell Movements, from Molecules to Motility.* Garland, New York.

Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001). Transmembrane extracellular matrix–cytoskeleton crosstalk. *Nat. Rev. Mol. Cell Biol.* **2**, 793–805.

Kaverina, I., Krylyshkina, O., and Small, J. V. (2002). Regulation of substrate adhesion dynamics during cell motility. *Int. J. Biochem. Cell Biol.* **34**, 746–761.

Martin, P. (1997). Wound healing – aiming for perfect skin regeneration. *Science* **276**, 75–81.

Martin, P., and Wood, W. (2002). Epithelial fusions in the embryo. *Curr. Opin. Cell Biol.* **14**, 569–574.

Montell, D. J. (2003). Border-cell migration: the race is on. *Nat. Rev. Mol. Cell Biol.* **4**, 13–24.

Small, J. V., Geiger, B., Kaverina, I., and Bershadsky, A. (2002). How do microtubules guide migrating cells. *Nature Rev. Mol. Cell Biol.* **3**, 957–964.

Trinkaus, J. P. (1984). *Cells into Organs*. Prentice-Hall. Englewood Cliffs, NJ, USA.

Webb, D. J., Parsons, J. T., and Horwitz, A. F. (2002). Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. *Nat. Cell Biol.* **4**, E97–E100.

## Biography

Victor Small graduated from King's College in London in 1969 with a Ph.D. in Biophysics. From 1970 to 1977 he held a lectureship position in Aarhus University, Denmark where he began work on contractile systems and the cytoskeleton. Since 1977, he has headed the Department of Cell Biology of the Institute of Molecular Biology of the Austrian Academy of Sciences in Salzburg. His laboratory explores live cell microscopy in studies of the cytoskeleton and the mechanisms underlying cell motility.

Emmanuel Vignal is a postdoctoral Fellow at the Institute of Molecular Biology in Salzburg. He graduated in 2001 with a Ph.D. in Cell Biology from the University of Montpellier in France. His current work focuses on the molecular mechanisms of lamellipodia protrusion.

# Cell–Matrix Interactions

**Janet A. Askari and Martin J. Humphries**
*The Wellcome Trust Centre for Cell–Matrix Research, School of Biological Sciences, University of Manchester, Manchester, UK*

**The extracellular matrix (ECM) is a network of macromolecules that underlies all epithelia and endothelia and that surrounds all connective tissue cells. The ECM provides mechanical support and also profoundly influences the behavior and differentiation state of cells in contact with it. The main receptors mediating the interaction of cells with ECM proteins are known as integrins, referring to their function of integrating the cell's exterior with its interior. Following ligand binding, the cytoplasmic domains of integrins connect to the cytoskeleton and trigger the assembly of signaling complexes. Conversely, the binding of intracellular cytoplasmic components influences cell adhesiveness by altering integrin conformation. Thus, a large variety of complex signaling events can be transduced by integrins in a bidirectional manner across the cell membrane. These events serve to modulate and coordinate many aspects of cell behavior, such as proliferation, survival, shape, polarity, motility, gene expression, and differentiation, that are required for such fundamental processes as development, tissue morphogenesis, and wound healing within multicellular organisms. Integrins are also implicated in several disease processes such as inflammation, thrombosis, and cancer metastasis, whereas mutations in integrin genes lead to deficiencies in leucocyte adhesion, myopathy, and blistering skin diseases.**

## The Extracellular Matrix

### Main Components

The extracellular matrix (ECM) components are diverse in composition, but they generally comprise a mixture of fibrillar proteins, polysaccharides, and glycoproteins synthesized, secreted, and organized by neighboring cells. Collagens, fibronectin, and laminins are the principal components involved in cell–matrix interactions; other components, such as vitronectin, thrombospondin, and osteopontin, although less abundant, are also important adhesive molecules.

Over 20 different collagens exist in mammals, but the most abundant exist as helical molecules that assemble into fibrils and provide tensile strength for many tissues. The ECM also contains a large number of glycoproteins, the best studied of which is fibronectin, a dimeric molecule that also forms insoluble fibrils. The laminins are heterotrimeric glycoproteins that fold into cruciform-shaped molecules and are important components of basement membranes. Fibrinogen and von Willebrand factor are also considered to be matrix proteins because they function as major adhesive molecules in blood, but they can also pass into extravascular fluid and influence cell function.

### Adhesive Motifs

Although matrix proteins are large molecules, their major integrin recognition sites are very short peptide motifs of only three to six amino acids. The best known and most widespread of these adhesive motifs is arginine–glycine–aspartic acid (RGD, using the single-letter amino acid nomenclature). This motif was first discovered in 1984 by Pierschbacher and Ruoslahti in the center of fibronectin (FN), but it is also present and functional in vitronectin, von Willebrand factor, and thrombospondin. The crystal structure of the area of FN containing this motif has been solved and shows the tripeptide extending out as a loop from the surface of the protein. Similar short peptide motifs are present in other matrix proteins (see Table I). A constant feature of the motifs is the presence of an acidic residue, either aspartic or glutamic acid, which is an absolute requirement for the adhesive activity of the proteins in which they reside. As yet, no corresponding motif has been determined in laminin; however, integrin binding has been localized to distinct regions within the molecule.

In FN, a second region of the molecule, termed the synergy site, has been shown to work in concert with the RGD motif and to enhance integrin-binding affinity. Part of the synergy site is a pentapeptide proline–histidine–serine–arginine–asparagine (PHSRN). Although they probably exist, no synergy sequences have as yet been defined for other matrix proteins.

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Adhesive Sequences in Matrix Proteins and their Integrin Receptors**[a]

| Matrix protein | Adhesive sequence | Integrin receptor |
|---|---|---|
| Collagens | GFOGER | $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, $\alpha11\beta1$ |
| Fibronectin | RGD | $\alpha5\beta1$, $\alpha V\beta3$, $\alpha8\beta1$, $\alpha V\beta1$, $\alpha V\beta6$, $\alpha IIb\beta3$ |
| | LDV | $\alpha4\beta1$, $\alpha4\beta7$ |
| | REDV | $\alpha4\beta1$ |
| Laminins | E1′ fragment | $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ |
| | E8 fragment | $\alpha3\beta1$, $\alpha6\beta1$, $\alpha7\beta1$, $\alpha6\beta4$ |
| Vitronectin | RGD | $\alpha V\beta3$, $\alpha IIb\beta3$, $\alpha V\beta5$, $\alpha V\beta1$ $\alpha V\beta8$ |
| Fibrinogen | RGD | $\alpha V\beta3$ |
| | KQAGDV | $\alpha IIb\beta3$ |
| von Willebrand factor | RGD | $\alpha IIb\beta3$, $\alpha V\beta3$ |

[a] GFOGER, glycine–phenylalanine–hydroxyproline–glycine–glutamic acid–arginine; KQAGDV, lysine–glutamine–alanine–glycine–aspartic acid–valine; LDV, leucine–aspartic acid–valine; REDV, arginine–glutamic acid–aspartic acid–valine; RGD, arginine–glycine–aspartic acid.

# Integrins

## Structure

Integrins are noncovalently linked dimers consisting of an $\alpha$- and a $\beta$-subunit, and the 18 $\alpha$- and 8 $\beta$-subunits that have been identified in humans combine to form 24 different receptors. Integrin homologues are present in organisms ranging from sponges to humans, indicating their central role in metazoan evolution. Integrins possess large extracellular domains, approximately 1200 amino acids in $\alpha$-subunits and 800 in $\beta$-subunits, a transmembrane domain, and short cytoplasmic regions of 50 residues or less. The exception to this rule is the $\beta4$-subunit, which has a cytoplasmic domain of over 1000 amino acid residues. In addition, half of all $\alpha$-subunits contain an extra 200 residue module toward their N terminus (see Table II), which has homology with the von Willebrand factor A-domain and is referred to as the $\alpha$ A- or I- (for inserted) domain. Several $\alpha$ A-domains have been crystallized and all adopt a Rossmann fold characterized by central $\beta$-sheets surrounded by $\alpha$-helices. A conserved motif, the metal-ion-dependent adhesion site (MIDAS), which includes the sequence aspartic acid–any residue–serine–any residue–serine (DxSxS), coordinates a divalent metal cation at the top of the domain. The N terminus of the $\beta$-subunit also contains an A-domain with a MIDAS motif. Electron micrographs show the integrin dimer to have a globular head 8–12 nm in diameter that constitutes the ligand-binding domain from which two stalks 2 nm thick and 14–20 nm in length project; the distal regions of these contain hydrophobic sequences representing the transmembrane domains.

**TABLE II**

**Properties of Integrins**[a]

| Integrin | Alternative names | Matrix ligands |
|---|---|---|
| With an A-domain | | |
| $\alpha1\beta1$ | VLA 1, CD49a/CD29 | CO, LM |
| $\alpha2\beta1$ | VLA 2, GPIaIIa, CD49b/CD29 | CO, LM |
| $\alpha10\beta1$ | | CO, LM |
| $\alpha11\beta1$ | | CO |
| $\alpha D\beta2$ | CD11d/CD18 | [b] |
| $\alpha L\beta2$ | LFA-1, CD11a/CD18 | [b] |
| $\alpha M\beta2$ | Mac1, CD11b/CD18 | FG |
| $\alpha X\beta2$ | p150,95, CD11c/CD18 | FG |
| $\alpha E\beta7$ | $\alpha_{IEL}\beta7$, HML-1 antigen, CD103($\alpha$E) | [b] |
| Without an A-domain | | |
| $\alpha3\beta1$ | VLA 3, CD49c/CD29 | LM |
| $\alpha4\beta1$ | VLA 4, CD49d/CD29 | FN, OP |
| $\alpha5\beta1$ | VLA 5, FNR, CD49e/CD29 | FN, OP |
| $\alpha6\beta1$ | VLA 6, CD49f/CD29 | LM |
| $\alpha7\beta1$ | | LM |
| $\alpha8\beta1$ | | FN, TN, NN |
| $\alpha9\beta1$ | | TN, OP |
| $\alpha V\beta1$ | CD51/CD29 | FN, VN |
| $\alpha IIb\beta3$ | GPIIbIIIa, CD41/CD61 | FN, FG, VN, vWF, Tsp |
| $\alpha V\beta3$ | VNR, CD51/CD61 | VN, FG, FN, vWF, Tsp |
| $\alpha V\beta5$ | | VN |
| $\alpha V\beta6$ | | FN |
| $\alpha V\beta8$ | | VN |
| $\alpha6\beta4$ | CD49f/CD104 | LM |
| $\alpha4\beta7$ | LPAM-1 | FN |

[a] CO, collagen; FG, fibrinogen; FN, fibronectin; LM, laminin; NN, nephronectin; OP, osteopontin; TN, tenascin; Tsp, thrombospondin; VN, vitronectin; vWF, von Willebrand factor.
[b] Involved in cell–cell interactions only.

Those $\alpha$-subunits that do not contain A-domains undergo posttranslational cleavage into a light and heavy chain held together by a disulfide bond, the exception being $\alpha4$, which is cleaved at a more central position to yield fragments of 70 and 80 kDa. In addition, several integrin subunits, $\alpha3$, $\alpha6$, $\alpha7$, $\beta1$, $\beta3$, $\beta4$, and $\beta5$, are subject to alternative splicing mainly in their cytoplasmic domains, yielding isoforms that are differentially expressed in specific patterns. This splicing serves to increase the cell–matrix interaction repertoire and hone the temporal and spatial adhesive responses of cells.

The field of integrin research was advanced enormously by the publication of the crystal structure of the extracellular domain of $\alpha V\beta3$ in 2001 by a group led by Arnaout. This structure shows that the globular head of

the integrin is made up of a seven-bladed $\beta$-propeller contributed by the $\alpha$-subunit and the A-domain from the $\beta$-subunit together with an immunoglobulin fold, termed the hybrid domain, made up of polypeptide sequences from either side of the $\beta$ A-domain. The integrin stalks are folded into three $\beta$-sandwich domains in the $\alpha$ and four epidermal growth factor (EGF)-like repeats in the $\beta$-subunit. The $\beta$-subunit also has an N-terminal plexin–semaphorin–integrin (PSI) domain and a novel cystatin-like fold just before the transmembrane region. An arginine residue, R261, in the $\beta$3-subunit A-domain extends into the core of the $\alpha$-subunit propeller, where it is held in place by aromatic residues and is the main area of subunit association.

### Ligand Binding

All interactions between integrins and matrix proteins are dependent on divalent cations, whereas the ligand-binding specificity of integrin molecules is determined by the particular $\alpha$–$\beta$ combination. Some matrix proteins (e.g., FN) can bind to several integrins, or, conversely, one integrin can recognize ligands of diverse structure; for example, $\alpha2\beta1$ binds to both laminin and collagen. In general, each integrin has a specific nonredundant function, which is emphasized by the distinct phenotypes of knockout mice.

The integrin dimers can be broadly divided into three families consisting of the $\beta1$, $\beta2/\beta7$, and $\beta3/\alpha$V integrins. $\beta1$ associates with 12 $\alpha$-subunits and can be further divided into RGD-, collagen-, or laminin-binding and the related $\alpha4/\alpha9$ integrins that recognise both matrix and vascular ligands. $\beta2/\beta7$ integrins are restricted to leukocytes and mediate cell–cell rather than cell–matrix interactions, although some recognize fibrinogen. The $\beta3/\alpha$V family members are all RGD receptors and comprise $\alpha$IIb$\beta3$, an important receptor on platelets, and the remaining $\beta$-subunits, which all associate with $\alpha$V. It is the collagen receptors and leukocyte-specific integrins that contain $\alpha$ A-domains.

In non-$\alpha$ A-domain-containing integrins, both the $\alpha$- and $\beta$-subunits are required for ligand binding. An integrin–ligand complex crystal structure showed that RGD peptide binds at the $\alpha$–$\beta$ interface, with its arginine residue contacting the $\alpha$-subunit propeller and its aspartate helping to coordinate the divalent cation at the MIDAS site via a carboxyl linkage, which in the presence of ligand is occupied. Two further cations are present in the ligand-bound structure, one at a site adjacent to the MIDAS (termed ADMIDAS) and the other at a site in close proximity, termed the ligand-induced metal-binding site (LIMBS). Isolated recombinant $\alpha$ A-domains are able to bind peptide and macromolecular ligands with the same affinity as intact dimer. A crystal structure of the A-domain of $\alpha2$ in complex with a triple-helical collagenous peptide containing the glycine–phenylalanine–hydroxyproline–glycine–glutamic acid–arginine (GFOGER) motif also shows the carboxyl from an acidic residue, in this case glutamate, from the ligand directly completing the coordination sphere of the metal ion in the MIDAS. This explains the absolute requirement for either aspartic or glutamic acid in matrix adhesive proteins and the dependence on divalent cations for integrin–ligand interactions.

### Cation Modulation

The binding of ligand to integrin cannot take place without a divalent cation, which directly contacts a carboxyl group from the ligand. However, cations also play an important role in the regulation of integrin affinity. Integrin $\alpha$V$\beta3$ contains six cation-binding sites in the unliganded crystal structure and eight in the presence of cyclic RGD peptide, at least three of which are affected by ligand binding. In general, integrin–ligand binding is stimulated by magnesium and manganese ions and inhibited by calcium ions. However, this situation is complicated by the fact that the binding of one cation can affect the binding of another at a different site. In addition, several cation-binding sites within an individual integrin can influence ligand binding. The physiological relevance of cation modulation of integrin affinity has yet to be resolved.

## Activation and Signaling

### Focal Adhesions

When cells attach and spread on a matrix ligand, there is an initial clustering of integrins in the membrane, followed by an accumulation of cytoskeletal and signaling molecules into dynamic structures known as focal adhesions or focal contacts. These structures represent the anchor points of the cell where integrins link the cell to both the underlying matrix and the intracellular actin filaments of the cytoskeleton. The contacts appear as dense plaques, often located near the cell periphery, and provide both a scaffold and a means whereby cells can generate traction during migration. More important, focal adhesions also serve as nucleation points for the recruitment of not only structural proteins such as vinculin, talin, and paxillin, but also many signaling and adaptor molecules able to trigger a cascade of phosphorylation events that can activate numerous downstream targets.

### Conformational Changes

Many integrins are not constitutively active, and adhesion of cells to matrix proteins needs to be strictly

controlled and regulated in response to environmental changes. Integrins exist in at least three states, inactive, active, and ligand-bound; the switching of integrins from an inactive to an active state involves conformational changes not only in the ligand-binding pocket, but also across the whole of the extracellular domain and in the cytoplasmic face. Thus, the activation of the ligand-binding domain in the head and the binding of ligand are coupled via long-range conformational changes to signaling events in the cytoplasm. Conversely, intracellular events effect changes in the cytoplasmic domains that are translated in the opposite direction to the integrin head, allowing activation and ligand binding. This bidirectional communication is termed outside-in and inside-out signaling.

## Cytoplasmic Domains

The short cytoplasmic domains of integrins play a vital role in integrin function because they control the activation states of integrins and are required to maintain integrins in an inactive state. They are also the sites of interaction with, and linkage to, both cytoskeletal and signaling molecules when clustered in focal adhesions. Interactions between the $\alpha$ and $\beta$ tails occur by a salt bridge and hydrophobic and electrostatic contacts in the $\alpha$-helical, membrane-proximal region of both domains, and it is likely that this association is lost on integrin activation, allowing the tails to separate and effector molecules to bind. The $\beta$-subunit tail is the principal site for binding of cytoplasmic molecules, whereas the $\alpha$-subunit plays a more regulatory role in controlling activation.

Many proteins are intimately associated with integrins in focal adhesions, but direct interaction has only been proven for the binding of the structural protein talin to the $\beta 3$ cytoplasmic domain. The site of interaction is a conserved asparagine–proline–any residue–tyrosine (NPxY) motif in the $\beta 3$ tail with a phosphotyrosine-binding-like (PTB) subdomain of talin. This interaction may be the prototype for protein interactions with integrin $\beta$ tails through PTB domains.

## Signaling by Integrins

Integrin-mediated signaling can be broadly divided into two categories, direct and collaborative. In the first, ligation and clustering of integrins are the only stimuli and adhesion to ECM proteins activates cytoplasmic tyrosine kinases, for example, focal adhesion kinase (FAK) and serine/threonine kinases such as those in the mitogen-activated protein kinase (MAPK) cascade. Direct signaling also induces ionic transients (e.g. $Ca^{2+}$, $Na^{+}/H^{+}$) and stimulates lipid metabolism. In collaborative signaling, integrin–ECM adhesion modulates signaling initiated by other types of receptors, such as receptor tyrosine kinases (RTK) and G protein-coupled receptors, allowing cells to integrate positional information concerning matrix contacts with information about the availability of soluble growth or differentiation factors that are also located in the ECM. Cells that are deprived of anchorage to the ECM eventually die by a specialized form of programmed cell death (apoptosis) known as anoikis. Cell–matrix interactions via integrins are thus essential for cell survival. Integrins also directly affect the organization of the cytoskeleton, and consequently cell motility, by activating the Rho GTPases, a branch of the Ras GTPase superfamily, particularly CDC42, Rac1, and RhoA. Rho promotes the formation and maintenance of actin stress fibers, whereas Rac and CDC42 regulate structures such as lamellipodia and filopodia, respectively.

It is worth remembering that each signaling pathway mentioned influences others at some point, so signaling within a cell is better considered as a series of networks rather than a direct path. Thus, integrins are able to modulate, either directly or indirectly, every signaling pathway within the cell, a fact that emphasizes the importance of cell–matrix interactions on all aspects of cell behavior.

## See Also the Following Articles

Focal Adhesions • Integrin Signaling • Rho GTPases and Actin Cytoskeleton Dynamics

## Glossary

**extracellular matrix (ECM)** A network of proteins and polysaccharides underlying and surrounding cells.

**focal adhesions** Points of anchorage of the cell to the underlying matrix allowing communication between the inside and outside of the cell via integrins.

**integrins** A family of $\alpha\beta$ heterodimeric cell surface receptors for extracellular matrix proteins.

**inside-out signaling** The modulation of integrin–ligand binding activity by intracellular events, leading to integrin clustering and conformational changes.

**outside-in signaling** The modulation of cell phenotype or behavior by extracellular events such as ligand binding.

## Further Reading

Humphries, M. J., McEwan, P. A., Barton, S. J., Buckley, P. A., Bella, J., and Mould, P. A. (2003). Integrin structure: Heady advances in ligand binding, but activation still makes the knees wobble. *Trends Biochem. Sci.* **28**, 313–320.

Hynes, R. O. (2002). Integrins: Bidirectional, allosteric signaling machines. *Cell* **110**, 673–687.

Xiong, J-P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L., and Arnaout, M. A. (2001). Crystal structure of the extracellular segment of integrin $\alpha V\beta 3$. *Science* **294**, 339–345.

## BIOGRAPHY

Janet Askari is a researcher in the Wellcome Trust Centre for Cell–Matrix Research at the University of Manchester, U.K., with an interest in mechanisms of signal transduction by integrins. She received a B.Sc. (Hons) Microbiology from the University of Kent in 1976.

Martin Humphries is Professor of Biochemistry, a Wellcome Trust Principal Research Fellow, and Director of the Wellcome Trust Centre for Cell–Matrix Research at the University of Manchester, U.K. His interests center on integrin structure, mechanisms of integrin priming and activation, and coordination of adhesion-dependent signaling. He received a B.Sc. (Hons) Biochemistry from the University of Manchester in 1980 and a Ph.D. in Biochemistry from the University of Manchester in 1983.

# Centromeres

**Beth A. Sullivan**
*Boston University School of Medicine, Boston, Massachusetts, USA*

**The centromere is a specialized mixture of DNA and proteins, and ensures chromosome inheritance and genome stability. As a chromosomal locus, the centromere is the minimal DNA or chromatin element that promotes formation of the proteinaceous kinetochore complex and coordinates chromosome movement in mitosis and meiosis. The centromere also synchronizes aspects of chromosome structure, such as heterochromatin formation, sister chromatid cohesion, and chromosome condensation. It is a multidomain locus, recruiting a variety of proteins with distinct functions.**

## Organization of the Centromere Region

Cytologically, centromeres have been defined by the visible primary constriction on metaphase chromosomes. This chromosomal locus is structurally complex, and contributes to various processes that ensure chromosome stability. The centromere region can be broadly classified into two major domains that encode kinetochore and heterochromatin functions. The kinetochore and heterochromatin are assembled independently, but each is equally important for complete centromere function and for ensuring chromosome and genome stability. The centromere/kinetochore domain comprises both DNA and proteins involved in chromatin assembly and structural aspects of the kinetochore. The heterochromatin domain is located adjacent to, or flanks, centromeric chromatin. Studies in various organisms have shown that heterochromatin is equally important for centromere function and chromosome inheritance as the kinetochore domain.

### Kinetochore Domain

At metaphase, the kinetochore, a proteinaceous multidomain structure, is assembled on the outer surface of the centromere, promoting attachment of the chromosome to spindle microtubules and movement during anaphase.

#### The Inner Kinetochore

The inner kinetochore is the region most intimately associated with centromeric DNA and/or chromatin. Many structural proteins that bind centromeric DNA or contribute to specialized chromatin structure are located here (Table I). The inner kinetochore contains constitutive proteins that serve as the foundation for the kinetochore, often termed the prekinetochore. CENP-A, a centromere-specific histone H3 variant that replaces H3 in centromeric nucleosomes, is located here and serves as an initiatory signal for kinetochore assembly by recruiting other inner and outer kinetochore proteins.

#### The Outer Kinetochore

The outer kinetochore region contains primarily microtubule-associated and chromosomal motor proteins that are involved in chromosome congression to the midzone at metaphase or engage spindle microtubules and move chromosomes to spindle poles in anaphase. In addition, the outer kinetochore contains surveillance or checkpoint proteins that monitor kinetochore attachments to the spindle and regulate the transition from metaphase to anaphase.

#### The Central Domain

The centromeric region that spans the interior of the centromere and connects sister kinetochores comprise the interior central domain. Centromeric DNA-binding proteins are concentrated here, as well as cohesion and condensation proteins and transiently associated proteins (chromosomal passengers) that co-ordinate chromosome segregation and cytokinesis.

### Heterochromatin Domain

Heterochromatin is cytologically dense material that is typically found at centromeres and telomeres. It mostly consists of repetitive DNA sequences and is relatively "gene poor." Its most notable property is its ability to silence euchromatic gene expression. Centromeres in yeast, fruit flies, and mammals are flanked by heterochromatin, indicating that its repetitive composition

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Homologous Centromere Region Proteins in Different Species**

| Location | Function | *S. cerevisiae* | *S. pombe* | *C. elegans* | *D. melanogaster* | *H. sapiens* |
|---|---|---|---|---|---|---|
| Kinetochore | Centromere specific histone | Cse4p | Cnp1 | HCP-3 | CID | CENP-A |
| | Centromeric chromatin architecture | | Mis6<br>Mis12<br>Mal2<br>Sim4 | | | CENP-I |
| | Inner plate: structure and DNA binding | Mif2p | Cnp3 | HCP-4 | | CENP-C<br>CENP-G<br>CENP-H |
| | Outer plate: chromosome congression and movement | CBF1, CBF3 | | HCP-1,2 | Cenpmeta<br>Cenpana<br>Zw10<br>Rod | CENP-E<br>CENP-F<br>ZW10<br>ROD |
| Heterochromatin | Histone H3 methyltransferase | | Clr4 | | Su(var)3-9 | SUVAR39H1 |
| | Heterochromatin formation | | Swi6<br>Chp1<br>Rik1 | | Su(var)2-5/HP1 HP2 | HP1 |
| | Sister chromatid cohesion | Scc1/Mcd1, Scc3<br>Pds5 | Rad21/Scc1<br>Mis4,6,12 | SCC-1/COH-2 SCC-3<br>EVL-14/PDS-5 | dRad21/Scc1 | RAD21/SCC1 |

or dense chromatin structure may represent an important, conserved function in centromere structure and function. Heterochromatin assembly is linked to chromatin regulation, occurring in a pathway that initiates with methylation of histone H3 at amino acid residue lysine 9 in order to recruit heterochromatin proteins (HPs), such as HP1 (heterochromatin protein 1). Once heterochromatin is established, cohesion and condensation proteins accumulate between sister kinetochores and chromatids. Mutations in HPs or certain histone modifying enzymes lead to chromosome mis-segregation and mitotic defects, indicating that heterochromatin contributes significantly to chromosome stability and segregation.

## Specification of Centromere Identity and Function

### The Centromere as DNA

How are the distinct kinetochore and heterochromatin domains assembled at centromeric regions? A well-debated question in centromere biology has been the role of primary DNA sequence in centromere identity and assembly. In a few organisms specific DNA sequences are required for centromeric protein binding, while in other organisms, centromere-specific DNA sequences have not been found. In addition, epigenetic mechanisms often determine centromere identity. In short, a centromere is established, and it is consistently maintained at a genomic region, but the site of formation is not determined by the underlying sequence itself. Recent studies indicate that RNA as well participates in centromere assembly.

#### Point Centromeres

Centromeres in the budding yeast *Saccharomyces cerevisiae* are the best studied and understood. *S. cerevisiae* centromeres are encoded by three distinct DNA elements (CDE I, II, and III) within a 125 bp region (Figure 1). Two elements (CDE I and III) are absolutely conserved and required to recruit centromere and kinetochore proteins. The centromeric histone CENP-A (Cse4p) is recruited to CDE II, the centromeric element that varies in sequence, but not size, from chromosome to chromosome. The small size of budding yeast centromeres, as well as the strict reliance on DNA–protein interactions for assembly, have resulted in detailed molecular maps and models of centromere assembly that are lacking in larger eukaryotes.

#### Regional Centromeres

Centromeres in fission yeast, *Schizosaccharomyces pombe*, and in flies, worms, mammals, and plants are monocentric, forming on a limited, specific region of the chromosome (Figure 1). These centromeres are comprised of multiple subunits encoding kilobase or megabase-sized genomic regions. Unlike in budding yeast, centromeric DNA sequences are not obviously conserved, and centromere identity and assembly depend largely on epigenetic factors.

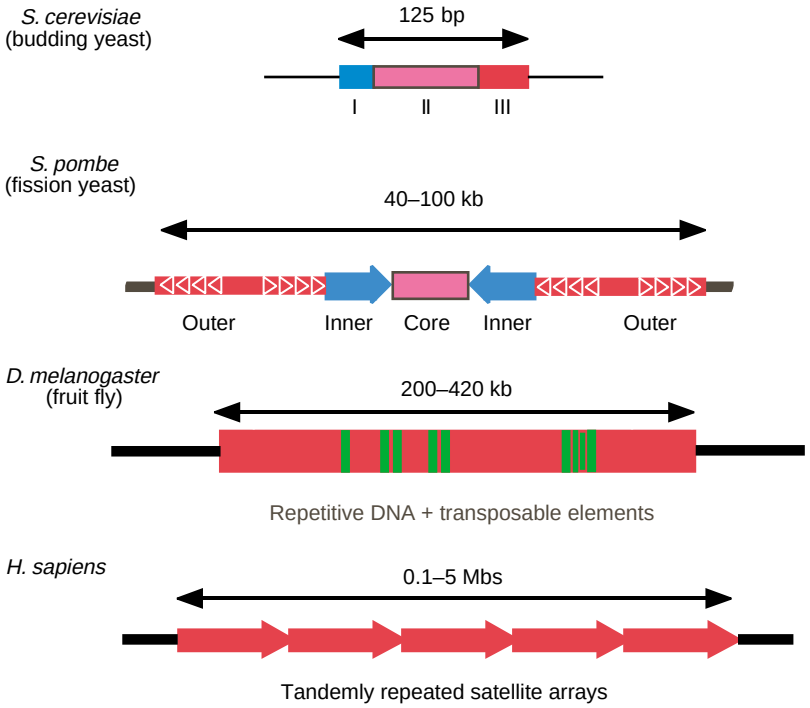



**FIGURE 1** Centromeric structure in various eukaryotes. Schematic diagram of eukaryotic centromeres. The DNA sequence of centromeres differs between species, but the organization of the centromere and the presence of centromere proteins is conserved. Budding yeast (*S. cerevisiae*) centromeres are 125 bp and are composed of three distinct elements, two of which are conserved (I and III). *S. pombe* (fission yeast) centromeres contain a unique central core flanked by inverted inner and outer repeats. *Drosophila* centromeres extend for 200–420 kb and contain repetitive DNA (red boxes) that is interspersed with transposable elements (green lines). Human centromeres consist of tandemly repeated alpha-satellite DNA arranged into higher order repeats that extend over several megabases.

***Schizosaccharomyces pombe*** The three centromeres in fission yeast consist of unconserved central core sequences flanked by inverted arrays of inner and outer repeats. Both the central core and portions of the inner repeats are required for establishment and maintenance of centromere function and for recruitment of CENP-A (Cnp1) and other centromeric proteins. The outer repeats are essential for assembly of centric heterochromatin through histone modifications and noncoding RNAs.

***Multisubunit Centromeres: Flies, Humans, and Plants*** Metazoan and plant centromeres are large, often ranging in size from a few hundred kb to 5 Mb. They primarily consist of thousands of copies of satellite DNA, often arranged in tandem (mammals and plants). In flies and plants, these repeats are often interspersed with transposable elements. In humans, *de novo* centromere assembly via artificial chromosomes has been most efficiently achieved when centromeric satellites (alpha-satellite DNA) are introduced into cells. However, not all human alpha-satellite DNAs form centromere *de novo*, indicating that other sequences or factors are required to assemble and maintain human centromeres.

***Holocentric Chromosomes*** In the nematode *Caenorhabditis elegans*, crayfish, and some insects, the holocentric chromosomes assemble centromeres along their lengths. *C. elegans* centromeres are the best-studied holocentrics. Most of the known eukaryotic centromere and kinetochore proteins have been identified in worms and the order of assembly, with CENP-A (CeCenp-A or HCP-3) at the top of the assembly pathway, are conserved. Proteins are recruited into distinct foci at prophase, but by metaphase, these foci spread evenly into "ribbons" along the poleward face of the chromosome arms. The distribution of centromere and kinetochore proteins into inner and outer kinetochore regions is conserved in *C. elegans*.

## The Centromere as Chromatin

Chromatin is an important regulator of gene expression and compartmentalization of the genome in interphase. Establishment of distinct chromatin domains (kinetochore, heterochromatin) is necessary for complete centromere function, thus chromatin may be a more important determinant of centromere function than DNA sequence.

### Epigenetic Centromeres

The simple presence of centromeric DNA in a cell or a chromosome does not necessarily correlate with centromere function. Besides the obvious lack of sequence conservation at eukaryotic centromeres, additional evidence exists to argue the role of underlying DNA sequence in centromere assembly. First at normal mammalian centromeres, only a fraction of the arrays actually participate in centromere assembly. In humans, the kinetochore domain typically comprises one-half to two-thirds of the satellite DNA found at eukaryotic centromeres. The remainder of the satellite DNA is involved in forming heterochromatin and cohesion. Secondly, naturally occurring or engineered dicentric chromosomes that contain two regions of centromeric DNA are stably transmitted in flies and humans only after inactivation of one centromere. Finally, neocentromeres are formed in flies and humans on stable marker chromosomes that completely lack centromeric sequences. None of the neocentromeres share sequence homology and even those that are derived from similar genomic regions assemble centromere, kinetochore, and heterochromatin proteins on different sequences.

### Specification by CENP-A

All centromeres are associated with the centromere-specific histone CENP-A. CENP-A is required to recruit all other centromere and kinetochore proteins, with the exception of HP1, making it is a strong candidate for a protein that specifies and maintains the site of kinetochore assembly. Unlike replication-dependent histones, CENP-A is dispersed in a semiconservative manner, thus providing the scaffold on which new CENP-A can be loaded and the centromere can be maintained irrespective of underlying DNA sequence. The organization of

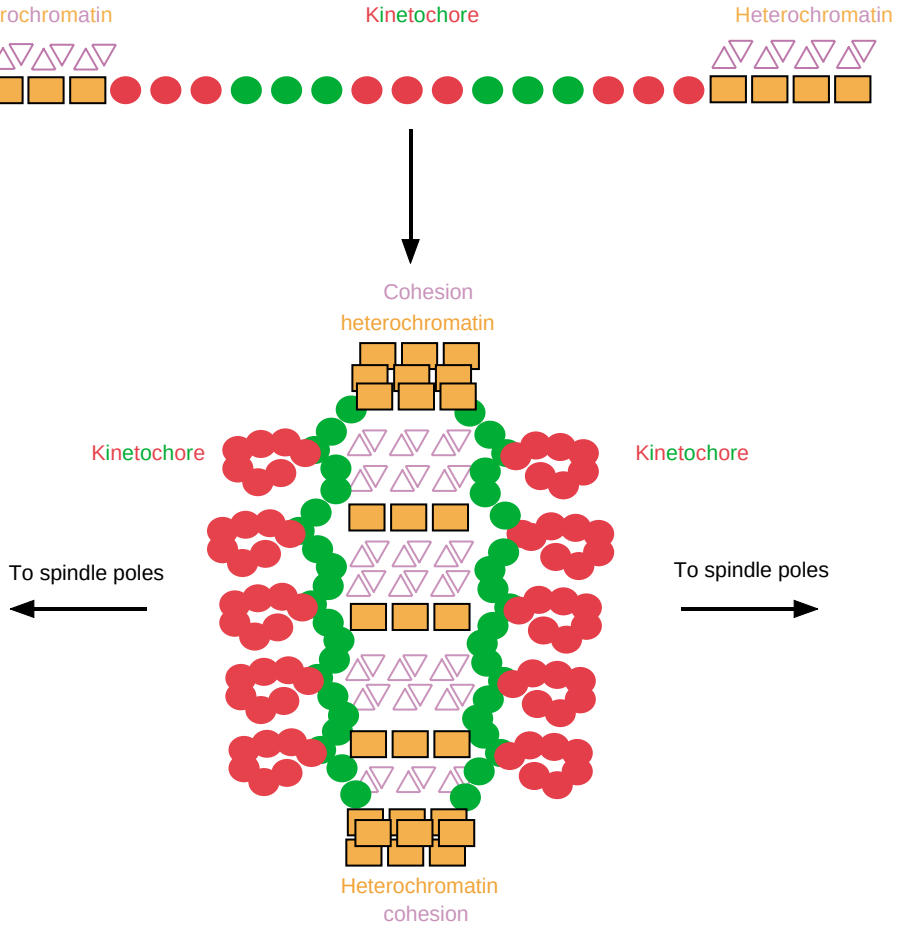



**FIGURE 2** Unique organization of centromeric chromatin creates proper structure of the centromere region. In 2D, CENP-A (red circles) and H3 (green circles) nucleosomes within centromeric chromatin are interspersed, forming the foundation of the kinetochore domain, which is flanked by heterochromatic proteins (squares) and cohesion proteins (triangles). In metaphase (3D), the blocks of CENP-A and H3 nucleosomes may be arranged in a spiral, orienting CENP-A nucleosomes to the poleward face of the chromosome, presumably to bi-orient the chromosome and facilitate recruitment and interactions with other kinetochore proteins. H3 nucleosomes are sequestered to the region between sister kinetochores, where heterochromatin and cohesion proteins are recruited. Flanking heterochromatin that is assembled between sister kinetochores may be as important as CENP-A:H3 interspersion for ensuring that CENP-A opposes spindle poles.

centromeric chromatin may further facilitate its maintenance and/or function. In two dimensions (2D), centromeric chromatin in flies and humans contains interspersed subunits of CENP-A and H3 nucleosomes (Figure 2). CENP-A subunits coalesce to form a three-dimensional (3D) interface that promotes recruitment of additional kinetochore proteins, biorientation of chromatids to opposite spindle poles, and attachments to microtubules. Conversely, the H3 subdomains are oriented toward the interior of the chromosome to establish a platform for recruiting cohesion and condensation proteins. The unique interspersion of CENP-A and H3 nucleosomes is important for centromere function, since depletion of CENP-A by RNA interference (RNAi) or in CENP-A mutants alters the ratio of CENP-A:H3 and leads to chromosome segregation defects and mitotic arrest.

## The Centromere as RNA

One of the most exciting discoveries in the past few years has been the involvement of double-stranded RNA (dsRNA) in specifying chromosomal, and particularly centromere, function. Although originally considered "junk DNA," the arrays of repeats that surround active centromeres have been shown to be functionally significant. In particular, noncoding, antiparallel RNAs are transcribed from the outer centromeric repeats in *S. pombe*. These transcripts are typically unstable and are normally processed by RNAi machinery that mediates the formation of heterochromatin. Full-length double-stranded transcripts from the repeats are reduced into small interfering RNAs (siRNAs) by the protein Dicer, a member of the RNA-induced silencing complex (RISC). The siRNAs then initiate methylation of histone H3 at lysine 9 that subsequently recruits heterochromatin proteins. The siRNAs are only required for establishment, but not maintenance of heterochromatin, and although noncoding RNAs from regions that contain CENP-A have not been identified, it is tempting to speculate that kinetochore-specific transcripts might initiate *de novo* centromere assembly that is then maintained by retention of CENP-A and/or other proteins through many rounds of replication and division.

## See Also the Following Articles

Chromatin Remodeling • Chromatin: Physical Organization • Chromosome Organization and Structure, Overview • Metaphase Chromosome • Nuclear Organization, Chromatin Structure, and Gene Silencing • Nucleoid Organization of Bacterial Chromosomes

## Glossary

**epigenetic** Referring to or describing any heritable influence on chromosome or gene function that is not correlated with, or dependent on, a change in DNA sequence.

**heterochromatin** Cytologically defined regions of the genome that contain repetitive DNA (satellite DNA, transposable elements). A defining feature of heterochromatin is the ability to silence gene expression.

**kinetochore** The proteinaceous structure on each chromosome that is responsible for chromosomal attachment to and movement along spindle microtubules.

**RNA interference** A cellular defense mechanism for specific gene silencing that is induced by double-stranded RNAs that are processed into 21–23 nucleotide small interfering RNAs (siRNAs), causing degradation of homologous endogenous mRNA.

## Further Reading

Choo, K. H. A. (1997). *The Centromere*. Oxford University Press, New York.

Cleveland, D. W., Mao, Y., and Sullivan, K. F. (2003). Centromeres and kinetochores: From epigenetics to mitotic checkpoint signaling. *Cell* **112**, 407–421.

Karpen, G. H., and Allshire, R. C. (1997). The case for epigenetic effects of centromere identity and function. *Trends Genet.* **13**, 489–496.

Martienssen, R. A. (2003). Maintenance of heterochromatin by RNA interference of tandem repeats. *Nat. Genet.* **35**, 213–214.

Mellone, B. G., and Allshire, R. C. (2003). Stretching it: Putting the CEN(P-A) in centromere. *Curr. Opin. Genet. Dev.* **13**, 191–198.

Sharp, J. A., and Kaufman, P. D. (2003). Chromatin proteins are determinants of centromere function. *Curr. Top. Microbiol. Immunol.* **274**, 23–52.

Sullivan, B. A., Blower, M. D., and Karpen, G. H. (2001). Determining centromere identity: Cyclical stories and forking paths. *Nat. Rev. Genet.* **2**, 584–596.

Sullivan, K. F. (2001). A solid foundation: Functional specialization of centromeric chromatin. *Curr. Opin. Genet. Dev.* **11**, 182–188.

## Biography

Dr. Beth Sullivan is an Assistant Professor in the Department of Genetics and Genomics at Boston University School of Medicine. Her research interests are in eukaryotic centromere structure and function, including organization and regulation of centromeric chromatin. She holds a Ph.D. from the University of Maryland, Baltimore and received postdoctoral training at Case Western Reserve University (Cleveland) and The Salk Institute (La Jolla). Her laboratory focuses on developing new cytological and cell biology techniques to facilitate comparative studies of centromere organization in mammals and *Drosophila*.

# Centrosomes and Microtubule Nucleation

**Reiko Nakajima, Ming-Ying Tsai and Yixian Zheng**
*Carnegie Institution of Washington and Howard Hughes Medical Institute, Baltimore, Maryland, USA*

**In animal cells, the microtubule network consisting of the centrosome and the filamentous microtubule array is required for intracellular trafficking, intracellular organization, and cell division. The timely assembly and organization of different microtubule arrays is critical for executing all functions of microtubules. Centrosome-mediated microtubule nucleation plays an important role in the formation of a functional microtubule network.**

## Microtubules

### Structure of Microtubules

Microtubules are assembled from dimers of $\alpha$- and $\beta$-tubulins that share significant ($\sim$50%) amino acid identity. Each tubulin has a molecular mass of $\sim$50 000 Da and binds to one molecule of GTP. Since the GTP-binding pocket of $\alpha$-tubulin is covered by $\beta$-tubulin in a tubulin dimer, only the GTP in $\beta$-tubulin can be exchanged and hydrolyzed. The tubulin dimers, often referred to as tubulin(s), polymerize in a head-to-tail fashion to form protofilaments that associate laterally into hollow cylindrical microtubules with diameters of $\sim$25 nm (Figure 1). The microtubule polymer exhibits a distinct polarity with a fast-growing plus end and a slow-growing minus end. Studies have shown that the plus and minus ends of microtubules terminate with $\beta$-tubulins and $\alpha$-tubulins, respectively.

### Microtubule Polymerization

Microtubule polymerization begins with a slow nucleation step where several tubulins come together to form a microtubule "seed" or "nucleus." The rate of microtubule nucleation is proportional to the $n$th power of tubulin concentration, where $n$ is the number of tubulins in a microtubule seed. Following nucleation, microtubule elongation by adding tubulins to the seeds occurs at a rate that is proportional to tubulin concentration. Therefore, microtubule nucleation is kinetically less favored than microtubule elongation. Once formed, the microtubule polymer exhibits a stochastic growth and shrinkage behavior termed "dynamic instability," which is defined by the rates of elongation and shrinkage as well as by the frequencies of transition from growth to shrinkage and from shrinkage to growth. Therefore, microtubule formation is governed by both the microtubule nucleation rate and by the various parameters of microtubule dynamic instability. These intrinsic features of microtubule polymers are exploited in living cells to achieve proper microtubule organization in response to various cellular signals.

## Centrosomes

### Structure of the Centrosome

The centrosome provides a major microtubule-nucleating and -organizing site in animal cells. Electron micrographs reveal that a centrosome consists of a pair of centrioles and an electron-dense pericentriolar material (PCM). Each centriole is barrel-shaped with nine triplet microtubules making up the barrel wall (Figure 2). Studies have shown that the two centrioles in a centrosome are linked by filamentous structures. An interesting feature of the centriole pair is that they are arranged in angles of varying degrees with respect to each other. The bottom of one centriole always faces the wall at one end of the second centriole that has additional appendages at the other end of the wall (Figure 2). Compared to the structure of centrioles, the structure of the PCM is less defined. With low-resolution electron microscopy, the PCM appears unstructured. Recent high-resolution electron tomography studies suggest that the PCM is made of filamentous matrices. Although the detailed organization of the centrosome matrix remains largely unknown, a number of centrosome proteins that have the potential to form a filamentous-matrix structure have been identified.

 © 2004, Elsevier Inc. All Rights Reserved. 

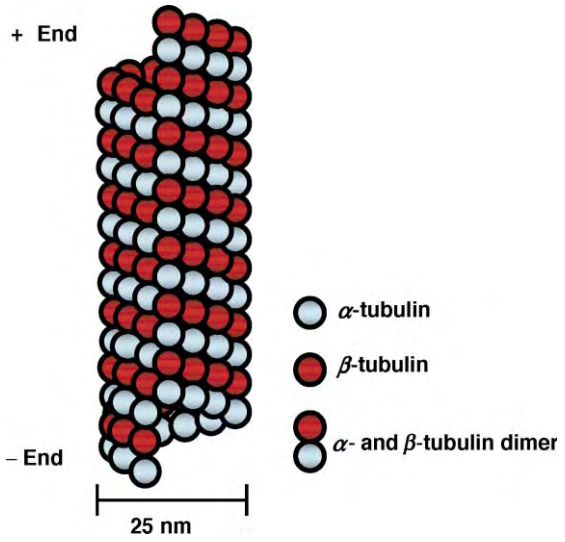



**FIGURE 1** Microtubules are assembled from α- and β-tubulin heterodimers. The dimers interact with each other in a head-to-tail fashion to form linear protofilaments that associate with each other laterally to form a hollow cylindrical microtubule with a diameter of 25 nm. The plus and minus ends of the microtubule are indicated.

### Centrosome Duplication

Unlike many other cellular organelles, a cell contains either one or two centrosomes depending on the cell-cycle stage. In G1 phase, there is only one centrosome per cell, which duplicates itself in S phase when the nuclear DNA is replicated. Centrosome duplication is characterized by the growth of a new centriole from the side of each of the existing centrioles, which results in two pairs of centrioles. Following centriole duplication, the PCM is partitioned between the two centriole pairs, giving rise to two centrosomes in G2 phase. As the cell cycle progresses into mitosis, the two centrosomes are further separated from each other and each centrosome participates in the organization of the bipolar mitotic spindle. At the end of a cell cycle, cell division results in two new cells each consisting of a single centrosome. Since bipolar spindle assembly is essential for equal chromosome segregation, the single duplication of the centrosome in each cell cycle is important for proper cell division. Recent studies show that the cyclin-dependent protein kinase, CDK2, drives centrosome duplication in S phase. However, the duplication process and the mechanism that ensures only one round of centrosome duplication per cell cycle are not well understood.

### The PCM Harbors Microtubule Nucleating and Organizing Activity

The centrosome was first recognized as a microtubule-nucleating and -organizing center over 100 years ago. Studies in the 1990s have now established that a protein complex called the γ-tubulin ring complex (γTuRC), which resides in the PCM, is responsible for microtubule nucleation. Hundreds of γTuRCs at the PCM nucleate the growth of plus ends of microtubules. The minus ends of these microtubules often associate with the PCM through interactions with γTuRC and/or with other PCM proteins (Figure 2), though the detailed mechanism of this association is not clear. The ability of the centrosome to associate with numerous nucleated microtubules gives rise to an organized microtubule array, which is essential for many aspects of microtubule functions.

## Microtubule Nucleation

### γTuRC

In addition to α- and β-tubulins, eukaryotic cells possess a third tubulin called γ-tubulin. Unlike α- and β-tubulins, γ-tubulin associates with nontubulin proteins to form protein complexes that can nucleate microtubule assembly. γ-Tubulin is a major component of the γTuRC that has been purified and studied in *Xenopus* eggs, *Drosophila* embryos, and human tissue culture cells. In all cases, γTuRC appears as an open-ring structure with a diameter of ~25 nm consisting of approximately seven proteins. The best-characterized *Drosophila* γTuRC consists of at least seven proteins: γ-tubulin, Dgp71WD, Dgrips75, 84, 91, 128, and 163. Dgrip84, Dgrip91, and γ-tubulin, which are the most abundant proteins in *Drosophila* γTuRC, have homologues in all organisms examined to date. Some of the remaining less abundant components of *Drosophila* γTuRC also have homologues in other animal cells. Therefore, the structure and organization of *Drosophila* γTuRC are likely to represent general features of γTuRC in animal cells.

In *Drosophila* γTuRC, γ-tubulin interacts directly with Dgrip84 and Dgrip91 to form a γ-tubulin small complex (γTuSC) consisting of two molecules of γ-tubulin and one molecule each of Dgrip84 and Dgrip91. Multiple γTuSCs are assembled into one γTuRC. Although a detailed structural model of γTuRC is not available, a number of studies strongly suggest that γTuRC consists of a ring and a cap that covers one face of the ring (Figure 3). The ring is primarily made of γTuSCs, whereas some or all of the less abundant γTuRC components (Dgp71WD, Dgrips75, 128, and 163) makes up the cap. The uncapped side of the γTuRC ends with a ring of γ-tubulins that directly interacts with tubulin to mediate microtubule nucleation (Figure 4).

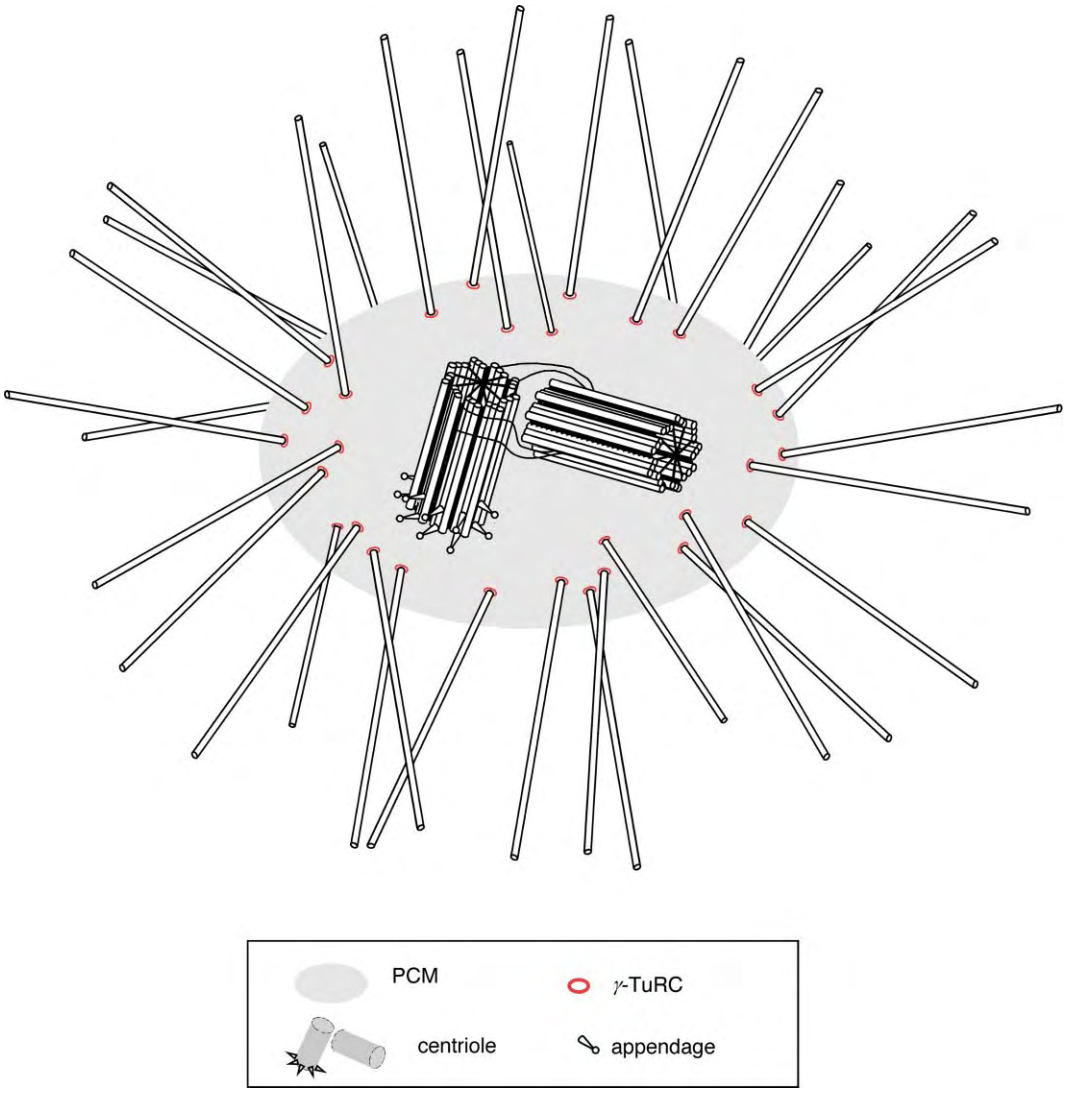



**FIGURE 2** The centrosome contains a pair of centrioles and a PCM. The centrioles are connected to each other and one of them has appendages. Microtubules are nucleated from γTuRCs that are associated with the PCM.

## Models of γTuRC-Mediated Microtubule Nucleation

The purified γTuRC can stimulate microtubule nucleation from purified tubulin *in vitro*. Although the detailed mechanism of γTuRC-mediated microtubule nucleation remains to be elucidated, two models have been proposed. Both models suggest that the presence of multiple γ-tubulins in the γTuRC allow simultaneous interactions with multiple tubulins during microtubule nucleation, thereby lowering the kinetic barrier of microtubule nucleation. However, the two models are completely different in terms of how the γTuRC may interact with its nucleated microtubules.

The so-called template nucleation model suggests that the γ-tubulin ring of the γTuRC acts as a template during microtubule nucleation (Figure 4). The newly formed microtubule is capped by the γTuRC at its minus end. Consistent with this model, a number of studies suggest that γTuRC-nucleated microtubules are capped at their minus ends. The other drastically different model—the protofilament model—suggests that the γTuRC ring opens up during microtubule nucleation with the multiple γ-tubulins acting as a protofilament to stimulate or stabilize the formation of microtubule seeds (Figure 4). Further high-resolution studies focusing on the interaction between the γTuRC and the microtubule minus ends are required to unequivocally discriminate between the two models of γTuRC-mediated microtubule nucleation.

## γTuRC-Mediated Microtubule Nucleation *in vivo*

In living cells, about half of the γTuRC is recruited to the centrosome where it mediates centrosome

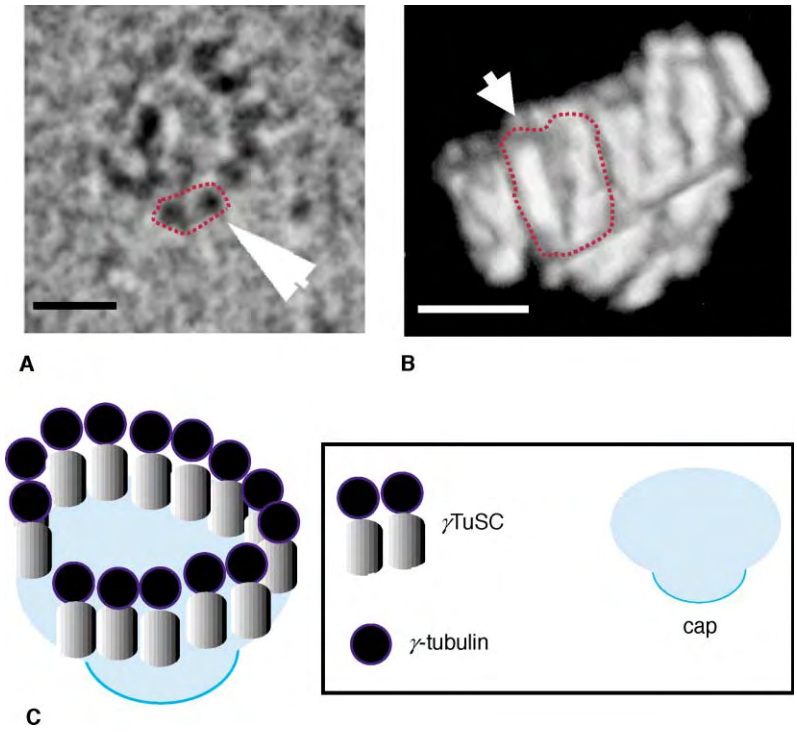



**FIGURE 3** The structure of the γTuRC. (A) Cryo-electron micrograph of a γTuRC in cross section. Approximately 13 subunits make up the ring. Arrow points to two subunits that may correspond to one γTuSC. The apparent internal structure may correspond to the cap. Reproduced from *J. Cell Biol.* **144**, 721–733, with permission. (B) Three-dimensional structure of a γTuRC reconstructed from negative staining samples. This side view of γTuRC shows the cap that covers one face of the ring. Arrow points to the structure that may correspond to one γTuSC. Reproduced from Moritz, M., Braunfeld, M. B., Guenebaut, V., Heuser, J., and Agard, D. A. (2000). Structure of the gamma-tubulin ring complex: A template for microtubule nucleation. *Nat. Cell Biol.* **2**, 365–370 with permision of Nature Publishing Group. (C) Schematic diagram of the γTuRC. The ring is primarily made of γTuSCs. Scale bars, 10 μm.

microtubule nucleation. Since centrosomes provide the major microtubule nucleating activity in dividing cells, it is not clear whether the other half of the γTuRC in the cytoplasm also participates in microtubule nucleation. One possibility is that the cytoplasmic γTuRC does not nucleate microtubules because it is associated with an inhibitor. Alternatively, centrosomal recruitment of γTuRC may greatly enhance its microtubule-nucleating activity. In this case, even though the cytoplasmic pool of γTuRC can nucleate microtubules, the centrosomal γTuRC provides the major source of microtubule-nucleating activity.

The cytoplasmic pool of γTuRC serves as a reserve for additional centrosome recruitment of γTuRC that occurs at the onset of mitosis. This recruitment of additional γTuRC contributes toward the enhanced microtubule-nucleating activity of the centrosome, which is important for efficient spindle assembly in mitosis. Interestingly, a fraction of the γTuRC also associates with the mitotic spindle in mitosis. It is possible that this pool of γTuRC plays a role in spindle assembly and function.

### Other Microtubule Nucleators

By definition, any proteins that facilitate the formation of microtubule seeds during microtubule nucleation can function as microtubule nucleators. Before the discovery of γ-tubulin and γTuRC, microtubule-associated proteins (MAPs) were considered as prime candidates for microtubule nucleators. MAPs may nucleate microtubules by binding and stabilizing tubulin oligomers formed during microtubule seed formation. Interestingly, some MAPs, such as XMAP215, are found at the centrosome in animal cells. Further study is required to determine whether or how the centrosomally localized MAPs mediate microtubule nucleation *in vivo*.

## Centrosome Abnormalities in Cancer

A number of studies have shown that abnormalities in centrosome shape, nucleating capacity, and number exist in cells derived from many tumor tissues. These centrosome anomalies can disrupt bipolar spindle assembly in mitosis, resulting in the formation of either monopolar or multipolar spindles and chromosome mis-segregation. Since aneuploidy – the gain or loss of chromosomes due to chromosome mis-segregation – is linked to genomic instability in human cancers, centrosome abnormalities have been implicated in causing aneuploidy and tumorigenesis.

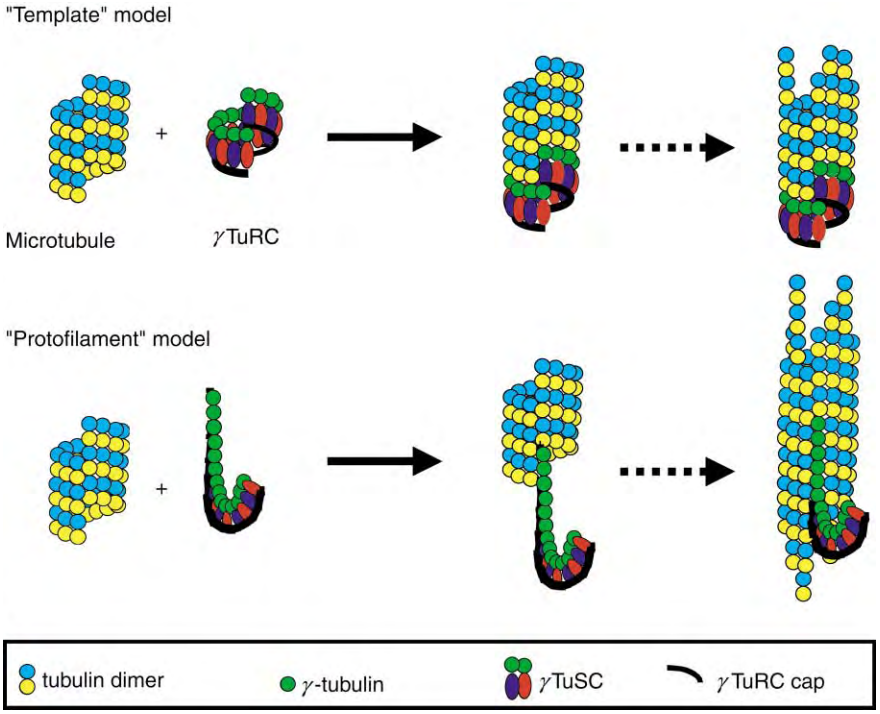



**FIGURE 4** Microtubule nucleation by the γTuRC. In the "template model" of microtubule nucleation, the γTuRC caps the minus ends of microtubules after nucleation. In the "protofilament model" of microtubule nucleation, the γTuRC undergoes a large structural change, resulting in the formation of protofilament-like γ-tubulins that nucleate microtubules. For simplicity, the cap of the γTuRC is represented as a line in the γTuRC drawings. (Reproduced from Wiese, C., and Zheng, Y. (2000). A new function for the γ-tubulin ring complex as a microtubule minus-end cap. *Nat. Cell Biol.* **2**, 358–364 with permission of Nature Publishing Group.)

## See Also the Following Articles

Chromosome Organization and Structure, Overview • Microtubule-Associated Proteins • Nucleoid Organization of Bacterial Chromosomes • Tubulin and its Isoforms

## Glossary

**aneuploidy** The state of the cell that has gained or lost a whole chromosome.

**bipolar spindle** A microtubule-based structure assembled during cell division, required for equal segregation of chromosomes into two daughter cells.

**chromosome mis-segregation** Inappropriate chromosome segregation that leads to gain or loss of chromosomes.

**genomic instability** The loss, gain, or rearrangement of chromosomes.

**tumorigenesis** Processes that lead to the development of cancer.

## Further Reading

Anderson, S. S. L. (1999). Molecular characterization of the centrosome. *Int. Rev. Cytol.* **187**, 51–109.

Desai, A., and Mitchison, T. J. (1997). Microtubule polymerization dynamics. *Annu. Rev. Cell Develop. Biol.* **13**, 83–117.

Keating, T. J., and Borisy, G. G. (2000). Immunostructural evidence for the template mechanism of microtubule nucleation. *Nat. Cell Biol.* **2**, 352–357.

Moritz, M., Braunfeld, M. B., Sedat, J. W., Alberts, B., and Agard, D. A. (1995). Microtubule nucleation by gamma-tubulin-containing rings in the centrosome. *Nature* **378**, 638–640.

Moritz, M., Braunfeld, M. B., Guenebaut, V., Heuser, J., and Agard, D. A. (2000). Structure of the gamma-tubulin ring complex: A template for microtubule nucleation. *Nat. Cell Biol.* **2**, 365–370.

Nigg, E. (2002). Centrosome aberrations: Cause or consequence of cancer progression? *Nat. Rev.* **2**, 1–11.

Oakley, B. R., Oakley, C. E., Yoon, Y., and Jung, M. K. (1990). γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell* **61**, 1289–1301.

Oegema, K., Wiese, C., Martin, O., Milligan, R., Iwamatsu, A., Mitchison, T., and Zheng, Y. (1999). Characterization of two related *Drosophila* γ-tubulin complexes that differ in their ability to nucleate microtubules. *J. Cell Biol.* **144**, 721–733.

Wiese, C., and Zheng, Y. (2000). A new function for the γ-tubulin ring complex as a microtubule minus-end cap. *Nat. Cell Biol.* **2**, 358–364.

Zheng, Y., Wong, M. L., Alberts, B., and Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. *Nature* **378**, 578–583.

## Biography

Yixian Zheng is a staff member and assistant investigator of the Carnegie Institution of Washington and the Howard Hughes Medical Institute, respectively. She holds a B. S. from Sichuan University (P. R. China) and a D.Phil. from the Ohio State University. Her research includes microtubule nucleation and mitotic spindle morphogenesis.

Reiko Nakajima holds a B.S. from Tokyo University and a D.Phil. from Osaka University, while Ming-Ying Tsai holds a B.S. from National Yang Ming Medical School and a D.Phil. from University of Southwestern Medical Center. Both are currently postdoctoral fellows in Yixian Zheng's group studying different aspects of spindle morphogenesis.

# c-*fes* Proto-Oncogene

**Thomas E. Smithgall**
*University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

**Robert I. Glazer**
*Georgetown University, Washington, DC, USA*

**The human c-*fes* proto-oncogene (Fes) encodes a protein-tyrosine kinase distinct from c-Src, c-Abl, and other non-receptor tyrosine kinases. Originally identified as the cellular homologue of avian (v-Fps) and feline (v-Fes) transforming retroviral oncoproteins, Fes is strongly expressed in myeloid hematopoietic cells where it may play a direct role in myeloid differentiation. The Fes promoter exhibits strong myeloid lineage specificity for directing transgene expression in animals. Fes is also expressed in endothelial and epithelial cells where it is involved in migration and cell survival, respectively. Structurally, Fes consists of a unique N-terminal region, a central SH2 domain, and a C-terminal kinase domain. Within the unique region are two coiled-coil oligomerization domains that regulate kinase activity. Fes is linked to growth, differentiation, and survival signaling through Ras and other small G proteins, STAT transcription factors, and phosphatidylinositol 3-kinase. Fes phosphorylates cell adhesion-related substrates in macrophages and promotes myelomonocytic differentiation. Fes is also involved in endothelial cell migration in response to angiogenic factors, neuron differentiation, and is up-regulated in the mammary gland during pregnancy. Although Fes had not been directly implicated in human disease, it can suppress the transforming activity of Bcr-Abl, the transforming oncoprotein associated with chronic myelogenous leukemia (CML).**

## Structure and Regulation

Human c-*fes* is located on chromosome 15 at position 15q26.1 and is comprised of 19 exons, the first of which is noncoding. The product of the *fes* gene is a 93 kDa protein-tyrosine kinase with three distinct structural regions (Figure 1). These include a long N-terminal unique region, a central Src homology 2 (SH2) domain, and a C-terminal kinase domain. Absent are an SH3 domain and signal sequences for lipid modification, such as an N-terminal myristoylation site associated with Src, Abl, and other nonreceptor tyrosine kinase families. Fes tyrosine kinase activity can be readily demonstrated *in vitro*, however, unlike its transforming viral counterparts, Fes kinase activity is tightly regulated in cells. As described, recent evidence suggests that the unique N-terminal region plays an important role in negative regulation.

### N-Terminal Region

One unusual feature of Fes is that its active form exists as a large oligomeric complex. Oligomerization requires the N-terminal region and promotes Fes autophosphorylation by a *trans* mechanism, a key step in the activation of the kinase domain. Analysis of the unique region using COILS, a computer algorithm that searches for the heptad repeat pattern associated with coiled-coil domains, reveals the presence of at least two regions with a high probability of forming coiled-coil structures. The presence of two N-terminal coiled-coil-forming sequences suggests several mechanisms for the regulation of Fes tyrosine kinase activity. One possibility is that a cellular protein binds to the coiled-coil regions and suppresses oligomerization. Alternatively, the two coiled coils may interact in an intramolecular fashion, thus preventing the formation of the active oligomer. A recent study has shown that Fes activity is repressed following ectopic expression in yeast, suggesting that the kinase naturally adopts the inactive conformation without the need for mammalian host cell factors. Deletion or mutagenesis of the more N-terminal coiled-coil domain releases the tyrosine kinase and biological activities of Fes in living cells, consistent with a critical role for this domain in negative regulation. In contrast, deletion of the more C-terminal coiled-coil domain impairs the kinase activity and biological function of active Fes mutants, suggesting a more dominant role in maintenance of the active state or recruitment of signaling partners.

In addition to its role in the regulation of kinase activity, the unique N-terminal region of Fes also contributes to the recruitment of signaling partners. One example is the breakpoint-cluster region protein (Bcr), which was originally discovered in the context of the Bcr-Abl oncoprotein associated with chronic

 © 2004, Elsevier Inc. All Rights Reserved. 

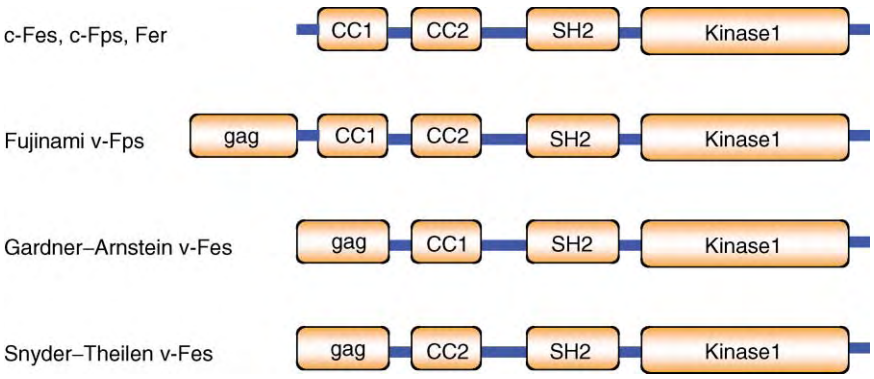



**FIGURE 1** Primary structure of the c-Fes protein-tyrosine kinase. Fes consists of a unique N-terminal region, a central SH2 domain, and a C-terminal kinase domain. Two coiled-coil oligomerization motifs (CC1 and CC2) are located in the unique region.

myelogenous leukemia (CML). Bcr is a multidomain signaling protein with an N-terminal Ser/Thr kinase domain, a central region with homology to the Dbl family of guanine nucleotide exchange factors, and a C-terminal GTPase-activating domain for Rho-related small GTPases. Bcr-Fes interaction involves the unique N-terminal region and SH2 domain of Fes and the N-terminal kinase domain of Bcr. Bcr is strongly phosphorylated by Fes both *in vitro* and *in vivo* on a cluster of sites localized to the N-terminal domain. Expression of this Bcr region suppresses Fes-induced neurite outgrowth in rat PC12 cells, suggesting that Bcr may couple Fes to Rho family small GTPases in neuronal cells (C. Laurent and T. Smithgall, unpublished data). Fes also interacts with Bcr-Abl through the Bcr-derived portion of the protein. This interaction may contribute to Fes-induced suppression of transformation signaling by Bcr-Abl.

### SH2 Domain

SH2 domains are signaling modules that mediate protein–protein interactions during tyrosine kinase signal transduction. By binding with high affinity and specificity to short, tyrosine-phosphorylated target sequences, SH2 domains mediate the assembly of multiprotein complexes in response to tyrosine kinase activation. Although the role of SH2 domains in the recruitment of effector proteins to autophosphorylated growth factor receptors is well known, SH2 domains were first identified within the sequences of nonreceptor tyrosine kinases. In fact, the SH2 domain of the Fes-related oncoprotein v-Fps was among the first to be described, and was shown to be required for full catalytic activity and to influence host range. Later work established a function for the SH2 domain in v-Fps-induced phosphorylation of transformation-related substrates. More recent studies reveal a similar dual role for the human Fes SH2 domain in the regulation of kinase activity and biological function. Deletion of the SH2 domain greatly reduces Fes kinase activity both in terms of substrate phosphorylation as well as autophosphorylation *in vitro*. The SH2 region also contributes to substrate recognition in macrophages.

### Kinase Domain

The Fes catalytic domain is localized to the C-terminal region of the protein and exhibits structural features typical of tyrosine kinases. These include a conserved lysine in the ATP-binding pocket (Lys 590) and a major site of tyrosine autophosphorylation (Tyr 713). Mutagenesis of Tyr 713 to Phe greatly reduces catalytic activity both *in vitro* and *in vivo*, suggesting that autophosphorylation is required for kinase activation. Substitution of Lys 590 with Glu or Arg completely abolishes kinase activity. *Trans* or autophosphorylation of Tyr 811 is likely associated with recruitment of other SH2 proteins but not with kinase regulation.

## Biological Functions

### Myeloid Differentiation

Early studies describing Fes expression patterns noted a striking correlation with terminal differentiation of myeloid hematopoietic cells. Fes expression is absent in myeloid leukemia cell lines resistant to myeloid differentiation inducers or selected for differentiation resistance, while expression increases following induction of differentiation with a variety of agents. Other work demonstrates a strong connection between Fes and the induction of myeloid differentiation. Chicken bone marrow cells infected with Fujinami sarcoma virus, which carries the fes-related oncogene, v-fps, undergo macrophage differentiation in the absence of macrophage colony-stimulating factor. Recent studies show

that expression of activated c-Fes mutants is sufficient to induce differentiation of myeloid leukemia cells along the macrophage lineage. Consistent with these observations, Fes activation is linked to a number of hematopoietic cytokines. Fes associates with the IL-4, IL-3, GM-CSF, and erythropoietin receptors. Kinase-inactive Fes blocks IL-4-induced phosphorylation of the insulin receptor substrate-2 and recruitment of phosphatidylinositol 3-kinase (PI3-K) in hematopoietic cells.

Evidence supporting a requirement for Fes in myeloid differentiation also comes from antisense experiments. Suppression of Fes expression in HL-60 promyelocytic leukemia cells using antisense oligonucleotides blocks the myeloid differentiation response to phorbol esters and other chemical inducers. However, targeted disruption of Fes in mice has yielded contradictory findings with respect to its role in hematopoiesis. Replacement of the *fes* alleles with a kinase-defective K588R mutant Fes did not show any remarkable effect on hematopoiesis, although STAT3 and STAT5A phosphorylation in response to GM-CSF was markedly reduced. In contrast to these findings, homozygous deletion of Fes led to a runted appearance, abnormal myeloid proliferation, but increased STAT3 activation in response to GM-CSF and IL-6. These apparently contradictory results can be explained by the competition of kinase-dead Fes with Jak2 for STAT3 resulting in a lower degree of STAT3 activation, whereas the complete absence of Fes produces less competition with Jak2 for STAT3 resulting in STAT3 hyperactivation.

### Is Fes a Tumor Suppressor?

Other work shows that Fes-induced differentiation may overcome signals for transformation in certain forms of leukemia. The first demonstration of this phenomenon involved transfer of the c-*fes* gene into the human cell line K-562, resulting in growth suppression and differentiation to macrophage-like cells. K-562 cells were established from the blast crisis phase of CML and exhibit no detectable Fes expression. Because they are CML derived, K-562 cells exhibit the Philadelphia chromosome translocation and express p210 Bcr-Abl, the oncogenic tyrosine kinase, that initiates the disease process. Thus, the Fes signal for differentiation is dominant to the Bcr-Abl signal for growth and survival. Fes can also suppress Bcr-Abl-induced transformation of murine myeloid leukemia cells to cytokine independence, providing further support for the idea that Fes may delay the progression of CML.

### Angiogenesis

In addition to its role in hematopoiesis, Fes may contribute directly to differentiation and development of the vascular endothelium. Expression of constitutively active Myr-Fes in transgenic mice under the control of the general SV40 promoter induced hypervascularity, which progressed to multifocal hemangiomas. This study also demonstrated expression of endogenous Fes in primary human vascular endothelial cells at levels comparable to those detected in myeloid cells. Consistent with these findings are the observations that Fes is activated by the angiogenic growth factors FGF-2 and angiopoietin-2 in capillary endothelial cells, and that overexpression of Fes is sufficient to induce formation of tube-like structures and chemotaxis in this cell type.

## Downstream Signaling Pathways

Major signaling pathways linked to Fes are briefly summarized here (Figure 2).

### Small G Proteins

Ras and other small *GTPases* are critical intermediates in most tyrosine kinase signal transduction pathways, and Fes is no exception. Activation of Ras and the related small GTPases Rac and Cdc42 are required for fibroblast transformation by Fes oncogenes. Dominant-negative mutants of all three small G proteins inhibit fibroblast colony-forming activity by Myr-Fes and v-Fps, and transformation correlates with constitutive activation of both the Erk and Jnk serine/threonine kinase pathways downstream. Sustained Erk activation is sufficient to induce myeloid differentiation of K-562 cells, suggesting that Fes-induced differentiation in these cells may be due in part to activation of the Ras/Erk pathway.

The activation and termination of small GTPase signaling are regulated by protein factors (guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively) that link small GTPases with upstream tyrosine kinases, including Fes and its transforming viral homologues. Transformation of fibroblasts by v-Fps has been shown to induce tyrosine phosphorylation of p120 Ras GAP, and similar results have been observed *in vitro* with purified Fes and GAP proteins. Fibroblast transformation by v-Fps also correlates with tyrosine phosphorylation of Shc, an adaptor protein that serves as an intermediate between tyrosine kinases and the Ras GEF complex, Grb-2/Sos. As mentioned above, endogenous Bcr is also strongly tyrosine-phosphorylated in fibroblasts transformed by v-Fps. Tyrosine phosphorylation of Bcr in v-*fps*-transformed cells induces its association with Grb-2/Sos via the Grb-2 SH2 domain. Like Shc, Bcr may serve as an intermediate between Fes and the activation of Ras downstream. In addition, tyrosine phosphorylation of

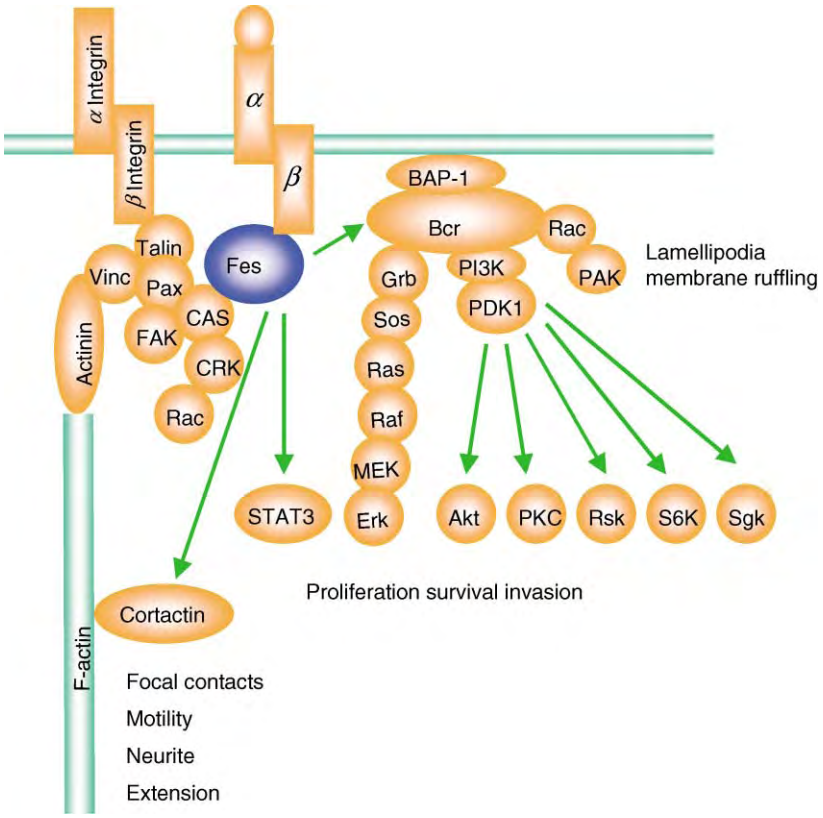



**FIGURE 2** Fes signaling pathways involved in cell motility, proliferation, survival, and differentiation.

Bcr may modulate either its GEF or GAP activities toward Rac and other GTPases.

## STAT Signaling

STATs are transcription factors with SH2 domains that are activated by tyrosine phosphorylation, often in response to cytokine stimulation. Although kinases of the Jak family are most often associated with STAT activation, a growing body of evidence indicates that Fes and other tyrosine kinases can induce STAT activation as well. For example, STAT3 DNA-binding activity is strongly activated following co-expression with Fes in Sf-9 insect cells, which lack endogenous Jak kinases. In addition, macrophages from homozygous knockout mice in which both *fes* alleles have been deleted hyperactivates STAT3 in response to GM-CSF. STAT3 has been strongly implicated in myeloid differentiation, making it tempting to speculate that Fes may signal to differentiation-related genes through a STAT3-dependent pathway.

## PI3-K Pathway

PI3-K phosphorylates phosphoinositol lipids at the 3′ position, leading to the generation of second messengers such as phosphatidylinositol-3,4,5-trisphosphate ($PIP_3$). PI3-K is a heterodimer consisting of 85 kDa regulatory (p85) and 110 kDa catalytic (p110) subunits, and interacts with tyrosine phosphorylated target proteins through SH2 domains found in the p85 subunit. Several reports have linked Fes to the activation of PI3-K signaling. Early work demonstrated elevated PI3-K activity in chicken embryo fibroblasts transformed by v-Fps as well as other transforming tyrosine kinases. More recently, IL-4 was shown to induce the association of endogenous Fes with PI3-K in IL-2-dependent T-cell lines. The SH2 domain of p85 forms a stable complex with Fes, suggesting a possible mechanism for factor-induced complex formation. The PI3-K pathway is often associated with survival signaling, and activation of this pathway by Fes may contribute to macrophage survival, perhaps through association with the Crk substrate, CAS (Figure 2). Interestingly, the protein-serine kinase immediately downstream to PI3-K, 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Figure 2), coimmunoprecipitates with Fes, and is phosphorylated on tyrosine (Zeng and Glazer, unpublished data). This suggests a mechanism by which Fes can mediate the activation of other downstream signaling pathways such as $p70^{S6K}$. Activation of PI3-K is also essential to Fes-induced neurite extension in PC12 cells, where it may serve to couple Fes to Rac activation in this system.

### Other Substrates

Most of the substrates reported for Fes have been identified using fibroblast transformation as a model system. However, overexpression of Fes in a macrophage cell line, which represents a physiological context, led to the phosphorylation of proteins related to integrin-mediated responses including adhesion and cell-cell contact. Interestingly, phosphorylation of Shc and other proteins associated with mitogenic responses have not been observed. A role for Fes in cell adhesion is further suggested by the interaction of the Fps SH2 domain with cortactin (Figure 2), which is also phosphorylated by Fes. These results suggest a mechanism for the localization of Fes with focal adhesions.

### Transcriptional Regulation of Fes

Transgene expression directed by the 13 kb genomic Fes locus reproduced the myeloid-specific expression pattern of the endogenous gene. The Fes promoter sequence spanning + 28 to +2523 (within exon 1 to intron 3) directed myeloid-specific expression of a heterologous gene in transgenic mice. Myeloid-specific expression has been attributed to a tissue-specific repressor at + 441 to +454, a Fes-specific transcription factor (FEF) binding to a *cis*-acting element at − 9 to − 4 and to transcription factors Sp1, PU.1, and Spi-1 binding to elements within the − 425 to +75 promoter sequence. Although these studies account for the myeloid-selective expression of Fes, they do not address Fes expression in other tissues, such as endothelial and epithelial cells, and its transient expression in embryonic tissues, such as the brain. Fes up-regulation occurs during neuronal differentiation and accelerates neurite outgrowth in PC12 cells. Fes also associates with and activates semaphorin3A that is associated with axonal guidance and neuronal outgrowth, further suggesting a role for Fes in brain development and differentiation.

## Conclusions

The c-*fes* proto-oncogene encodes a unique cytoplasmic tyrosine kinase involved in myelopoiesis, angiogenesis, CML progression and likely survival in mammary epithelial cells. Although the SH2 and kinase domains found in Fes share homology with other tyrosine kinase families, the N-terminal region is structurally distinct. The presence of coiled-coil oligomerization domains within the N-terminal region suggests a mode of kinase regulation and substrate recruitment that is unique to this tyrosine kinase family. Fes is associated with terminal differentiation in myeloid cells, as opposed to other tyrosine kinases that serve a predominant role in proliferation and survival. Work implicating Fes in the regulation of vascular endothelial cell migration suggests a role in tumor angiogenesis, and Fes may also serve a role in regulating cell survival and apoptosis during mammary gland differentiation, as well as neuronal differentiation.

### See Also the Following Articles

Fibroblast Growth Factor Receptors and Cancer Associated Perturbations • Hematopoetin Receptors • Inositol Phosphate Kinases and Phosphatases • JAK-STAT Signaling Paradigm • Ras Family • Small GTPases • Src Family of Protein Tyrosine Kinases • Syk Family of Protein Tyrosine Kinases • Tec/Btk Family Tyrosine Kinases

### Glossary

**GM-CSF** Granulocyte/macrophage-colony stimulating factor.

**promoter** The DNA sequence upstream to the transcription initiation site that directs expression of the gene.

**STAT** (signal transducers and activators of transcription) Transcription factors originally identified as mediating the transcriptional effects of interferon.

### Further Reading

Glazer, R. I., Smithgall, T. E., Yu, G., and Borellini, G. (1991). The role of the c-*fes* proto-oncogene protein-tyrosine kinase in myeloid differentiation. In *Advances in Regulation of Cell Growth* (J. Mond, J. C. Cambier and A. Weiss, eds.) Vol. 2, pp. 41–60. Raven Press, New York.

Greer, P. (2002). Closing in on the biological functions of FPS/FES and FER. *Nat. Rev. Mol. Cell Biol.* **3**, 278–289.

Kuriyan, J., and Cowburn, D. (1997). Modular peptide recognition domains in eukaryotic signaling. *Annu. Rev. Biophys. Biomol. Struct.* **26**, 259–288.

Pawson (1988). Non-catalytic domains of a cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. *Oncogene* **3**, 491–495.

Sawyers, C. L. (1999). Chronic myeloid leukemia. *N. Engl. J. Med.* **340**, 1330–1340.

Scheijen, B., and Griffin, J. D. (2002). Tyrosine kinase oncogenes in normal hematopoiesis and hematological disease. *Oncogene* **21**, 3314–3333.

Smithgall, T. E. (1998). Signal transduction pathways regulating hematopoietic differentiation. *Pharmacol. Rev.* **50**, 1–19.

Smithgall, T. E., Rogers, J., Peters, K. L., Li, J., Briggs, S. D., Lionberger, J. M., Cheng, H., Shibata, A., Scholtz, B., Schreiner, S., and Dunham, N. (1998). The c-Fes family of protein-tyrosine kinases. *Crit. Rev. Oncogenesis* **9**, 43–62.

Takashima Y., Delfino, F. J., Engen, J. R., Superti-Furga, G., and Smithgall, T. E. (2003). Regulation of c-Fes tyrosine kinase activity

by coiled-coil and SH2 domains: analysis with *Saccharomyces cerevisiae*. *Biochemistry* **42**, 3567–3574.

Yu, G., Smithgall, T. E., and Glazer, R. I. (1989). K562 leukemia cells transfected with the human c-fes gene acquire the ability to undergo myeloid differentiation. *J. Biol. Chem.* **264**, 10276–10281.

## BIOGRAPHY

Thomas E. Smithgall is a professor in the Department of Molecular Genetics and Biochemistry at the University of Pittsburgh School of Medicine. His principal research interests are in the structural organization and signaling mechanisms of cytoplasmic protein-tyrosine kinases. He holds a Ph.D. from the University of Pennsylvania and received his postdoctoral training at the National Cancer Institute, NIH with Dr. Robert I. Glazer.

Robert I. Glazer is a professor in the Department of Oncology at Georgetown University School of Medicine. His principal research interests are in the signal transduction pathways mediate by protein kinases involved in transformation. He holds a Ph.D. from Indiana University and received his postdoctoral training at Yale University with Dr. Alan C. Sartorelli.

# Chaperones for Metalloproteins

**Valeria C. Culotta and Edward Luk**
*Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA*

**Metal ions are not only essential co-factors for enzymes, but are also potentially toxic to cells. Therefore, the passage of metals inside a cell must be tightly regulated. A family of proteins has been identified that functions in the intracellular trafficking of copper ions to enzymes that require the metal for activity. These copper carriers are known as copper chaperones or metallochaperones. This article will highlight recent findings regarding the mechanism of copper chaperones.**

## Historical Background

Chaperones for metalloproteins are a small family of molecules that have evolved specifically to insert metal ion cofactors into enzymes or other proteins that rely on metals for function. All living cells, from bacteria to humans, harbor numerous metalloproteins that require metal ions such as zinc, copper, manganese, or iron to function. These metals either can serve in a structural role to help maintain the proper conformation of a protein or can actually carry out the chemistry that represents the protein or enzyme's catalytic function. Generally, each metalloprotein is only active with one particular metal ion type and, if fortuitously loaded with the wrong metal, the function of the protein is inhibited. Therefore, it is paramount that the right metal be delivered to the right metalloprotein and also that it be delivered at the right time. How this occurs in a regulated fashion eluded biochemists until the late 1990s, when a new paradigm for metal ion homeostasis emerged. It was discovered that many metalloproteins actually coexist with accessory molecules that ensure the acquisition of the proper metal ion. These accessory proteins have been termed metallochaperones or chaperones for metalloproteins. The term chaperone, first coined by Tom O'Halloran in 1997, was derived from Webster's definition of chaperone, "one delegated to ensure proper behavior." In this case, the one delegated is the metallochaperone, which ensures the proper behavior of the potentially promiscuous metal ion.

## The Concept of Metal Trafficking

There are a number of barriers that a metal ion must cross before it reaches its metalloprotein target inside the cell. First, the metal existing in the extracellular environment must traverse the plasma membrane. Metals by themselves cannot penetrate lipid bilayers and therefore rely on metal-transporter molecules to direct their movement across membranes. A variety of membrane transporters exist at the cell surface that act in the specific uptake of metals such as copper, zinc, iron, and manganese. Once inside the cell, some metal ions are delivered directly to proteins in soluble compartments (i.e., the cytosol); however, many metalloproteins reside in membranous organelles such as the mitochondria or secretory pathway, and as such the metal needs to cross a second membrane barrier. This again is accomplished by metal transporters that help translocate the ion across the lipid bilayer. Finally, at a site proximal to the metalloprotein target, the metal cargo is carried by a highly specific metal chaperone that helps insert the metal into the proper site of the metalloprotein. In essence, a metal ion does not freely move about the cell but rather is carried through a well-defined pathway involving the combined action of metal transporters and metal chaperones. This process of moving metals through defined pathways is commonly referred to as metal trafficking, and there are many such highways for the trafficking of metals to metalloproteins.

## Why the Need for Metal Trafficking?

The cell devotes a great deal of effort to ensure a highly regimented fate of a metal ion. Why not instead have the metal freely diffusible in the cell? Metalloproteins typically exhibit a very high affinity for their cofactor and are inherently able to self-activate with metals in a test-tube setting. However, the availability of metals inside cells is very limited. Although metals are essential for life, metals are also notoriously toxic and can cause much cellular damage. Therefore, all organisms have

 © 2004, Elsevier Inc. All Rights Reserved. 

evolved with a series of metal detoxification pathways that act to sequester metals and prevent them from reacting at biological sites. A classic example is the metal-binding metallothionein protein that acts as a sponge for these ions and precludes metals from accumulating in their free ionic form. In measurements that have been made in yeast and bacteria, not a single atom of copper or zinc is freely available in the cytoplasm of the cell, in spite of the micromolar quantities of metal that are accumulated. Intracellular metalloproteins therefore have the challenge of acquiring their cognate metal cofactor in an environment that is essentially a vacuum for metals. To overcome this dilemma, the metal ion chaperones spare a few ions from the metal detoxification pathways and ensure the safe delivery of metals to the active site of metalloproteins. Whereas proteins such as metallothioneins act to protect the *cell from the metal*, the metal chaperones act to protect the *metal from the cell*.

## Copper Metalloproteins as Models for Metal Trafficking

Much of what is currently known about metal chaperones and metal trafficking pathways stems from studies on copper. Because copper is a redox-active metal ion that can readily donate or accept electrons, it serves as an excellent catalytic cofactor for enzymes involved in oxygen chemistry. Compared to metalloproteins that bind iron or zinc, copper-containing enzymes are relatively few in number. However, an abundance of information is available on the metal-trafficking pathways relevant to this small subset of metalloenzymes.

In eukaryotes, copper is generally taken up into cells via a cell surface transporter, CTR1. This transporter was originally discovered in 1994 by Andrew Dancis through elegant genetic studies in yeast, and the human homologue was subsequently isolated by J. Gitschier in 1997. Following entry into the cell via CTR1, the copper ion can be shuttled through one of many distinct trafficking pathways, depending on its destination or fate. The copper is either subject to detoxification (a dead-end fate) or is selected for use by copper-containing enzymes. Three such utilization pathways for copper have been identified, and these specifically supply the metal to copper proteins in the Golgi, the cytosol, and the mitochondria (Figure 1).

There are a variety of enzymes that acquire copper in the Golgi compartment, and these are copper proteins destined for the cell surface or for export outside the cell. Examples include multicopper oxidases needed for iron transport (i.e., human ceruloplasmin), extracellular superoxide dismutases for anti-oxidant defense

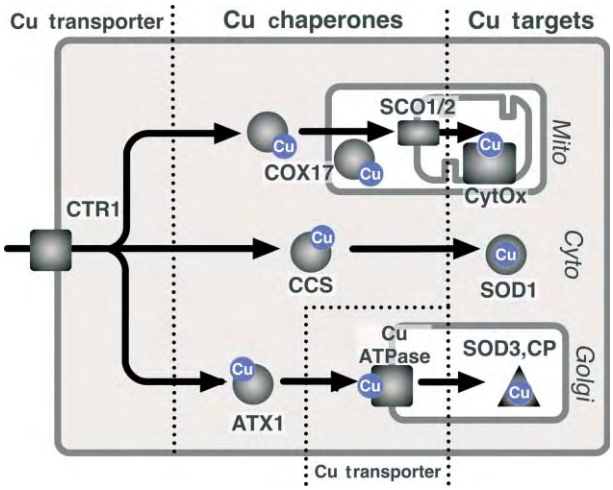



**FIGURE 1** Three metal-trafficking pathways involving copper in a typical eukaryotic cell. Copper is first taken up by the cell surface CTR1 copper transporter. The metal can then be delivered in one pathway involving cytosolic ATX1, which carries the copper to the P type copper-transporting ATPase. This transporter in turn pumps copper into the lumen of the Golgi for the activation of a variety of copper enzymes in the secretory pathway, such as extacellular superoxide dismutase (SOD3) and ceruloplasm (CP). A second pathway involves copper carried by CCS to the copper-zinc superoxide dimustase (SOD1) in the cytosol (Cyto). The third pathway involves a relay system of copper delivery to cytochrome oxidase (CytOx) in the mitochondria involving COX17 (both cytosolic and mitochondrial) and mitochondrial SCO1 and SCO2 proteins. Grouped within dotted lines are molecules designated as cell surface and intracellular copper transporters, copper chaperones, and targets for copper delivery.

(SOD3), enzymes for connective-tissue formation (lysyl oxidase), enzymes for pigment formation (tyrosinase), and enzymes for neuronal function (peptidly amidating enzyme and dopamine $\beta$-hydroxylase). Prior to reaching their location at the cell surface or extracellular milieu, all these enzymes pass through a specific compartment of the Golgi, where they are activated with copper.

A second copper-trafficking pathway in the cell delivers copper to the cytoplasm, where the ion activates a single enzyme, a cytosolic copper- and zinc-containing superoxide dismutase (SOD1). SOD1 functions to protect the cell against oxidative damage. The third copper-trafficking pathway delivers the metal to the mitochondria. Here a copper-containing oxidase (cytochrome oxidase) resides that functions as the terminal electron acceptor during cellular respiration.

These three pathways involve separate factors for transmembrane metal transport and metal ion chaperoning, and they are discussed independently next.

### Delivering Copper to the Golgi: Pathways Involving the ATX1 Copper Chaperone

In order for copper to gain access to enzymes that pass through the Golgi, the metal must traverse the

Golgi membrane. This is accomplished through the action of a specialized copper transporter, called P type ATPase, that uses the energy of ATP hydrolysis to drive the transmembrane translocation of copper from the cytoplasma into the lumen of the Golgi. Humans express two such copper transporters, known as the Wilson or Menkes disease proteins. As the names imply, these molecules have been associated with inherited diseases of copper transport. Copper-transporting ATPase molecules are found in virtually every eukaryotic cell, and they all contain a series of two to six short copper-binding protein domains that capture copper in the cytosol for translocation into the Golgi. How does the metal find these domains if there is no free copper in the cytosol? This is achieved through the action of a small copper-binding copper chaperone known as ATX1 (also called ATOX1).

ATX1 was originally identified in 1995 by V. Culotta as a potential anti-oxidant protein in yeast that guards against oxidative damage, hence the name ATX1. However, subsequent studies revealed that the major function of this molecule was to carry copper from the cytosol to the copper-transporting ATPase in the Golgi. Interesting, the polypeptide sequence of ATX1 is very similar to its recipient of copper delivery, the copper-binding domains of the Cu ATPase in the Golgi. ATX1 and these domains of the transporter bind copper via a single copper-binding site CXXC (where C = cysteine, a sulfur-containing amino acid, and X = any amino acid). With the same copper site present in the donor (ATX1) and recipient (Cu ATPase) molecules for copper transfer, copper is able to move from one site to the other by a facile exchange of sulfur ligands. This mechanism, originally proposed by O'Halloran, Culotta, and Penner-Hahn in 1997, was the first description of a copper-transfer reaction involving a copper chaperone.

## The Copper-Trafficking Pathway Involving the CCS Copper Chaperone for SOD1

The SOD1 enzyme is a copper- and zinc-containing superoxide dismutase that protects cells against oxidative damage by scavenging toxic superoxide anion radicals. This is a highly abundant copper enzyme that is ubiquitously expressed among eukaryotes, and in some cells SOD1 represents as much as 1% of the total cellular protein. It is the copper at the active site that carries out enzyme catalysis, whereas the zinc serves more of a structural role.

Although SOD1 has been found in numerous cellular compartments, the bulk ($\geq$95%) of the enzyme is cytosolic, and so there is no intracellular membrane barrier for copper delivery to SOD1. However, due to the absence of free cytsolic copper, SOD1 is highly dependent on its metal chaperone for acquiring its copper.

The copper chaperone for SOD1 (CCS) was first identified by Culotta and Gitlin in 1997. As is the case with ATX1, CCS was discovered by genetic studies in the baker's yeast *Saccharomyces cerevisiae*, but this protein is well conserved throughout eukaryotes. Unlike ATX1, which is a simple one-domain protein, CCS is a complex molecule consisting of three domains that serve separable functions in the copper-transfer process. At the extreme amino terminus, CCS has a short domain that exhibits extensive homology to ATX1, including the CXXC copper site. However, this site is not needed for copper transfer to SOD1 but rather appears to be involved in capturing copper under conditions of copper starvation. A large middle portion of CCS exhibits strong homology to SOD1, but is missing the SOD1 copper site. This homology allows CCS to dock with SOD1 by the formation of a transient heterodimer. SOD1 is normally a homodimer consisting of two identical molecules, and the formation of a heterodimer with CCS is the prerequisite to copper transfer. Finally, at the extreme carboxyl terminus of CCS lies a very short copper-binding domain containing a CXC copper site essential for mediating copper insertion into SOD1.

The mechanism of copper transfer from CCS to SOD1 is far more complicated than that described for ATX1. With ATX1, the donor and recipient for copper transfer bind the metal in the same manner. However, copper transfer by CCS is accompanied by a large change in metal coordination chemistry. Copper binds to CCS in the reduced Cu(I) form via sulfhydral ligands from cysteines in domain III and possibly in domain I as well. Yet in SOD1, copper is oxidized to the cupric Cu(II) state and is coordinated in an all-nitrogen environment. The mechanism by which transfer occurs with this drastic change in metal coordination is just now being elucidated. Oxidation of the copper ion is certainly involved, and oxygen or superoxide itself could be the driving force, as recently proposed by O'Halloran and colleagues.

## Delivery of Copper to Mitochondrial Cytochrome Oxidase

Cytochrome oxidase is a very large multisubunit enzyme that represents the terminal electron acceptor in the respiratory chain of the mitochondria. Two subunits of the enzyme contain harbor copper sites that are employed for electron-transfer reactions:

The $Cu_A$ site in subunit 2 of cytochrome oxidase contains two copper ions, whereas subunit 1 of the

enzyme harbors a single copper atom. The $Cu_A$ site acquires its metal via two sets of accessory proteins in the mitochondria: COX17 and SCOI/SCOII. Less is known regarding the assembly of the copper site in subunit 2, but it appears to require separate accessory factors. For the purposes of this review, we focus on assembly of the $Cu_A$ site.

As with ATX1 and CCS, all the accessory proteins for metal insertion into the $Cu_A$ site of cytochrome oxidase were originally discovered by genetic studies in the baker's yeast. Much of this work was pioneered by Moira Glerum and Alexander Tzagaloff in 1996.

COX17 is a cysteine-rich soluble copper-binding protein. Because COX17 exists in both the cytoplasm and the mitochondrial intermembrane space, it was proposed as the shuttle for copper ions between these cellular compartments. However, this function has not been confirmed and it is possible that COX17 only operates in the mitochondria to bring copper to the second set of accessory proteins for the $Cu_A$ site of cytochrome oxidase: SCO1 and SCO2.

The SCO proteins are two very homologous molecules that lie in the inner membrane of the mitochondria. These proteins are believed to capture copper from COX17 and then transfer the metal to the $Cu_A$ site of cytochrome oxidase. The SCO proteins clearly play an essential role in the assembly of cytochrome oxidase and in cellular respiration. A deletion of *SCO1* and also *COX17* in yeast results in a cell that is respiratory deficient. And in humans, mutations in *SCO2* have been associated with a fatal disease in infants, hypertrophic cardioencephalomyopathy.

## Perspectives

Virtually all that is known regarding specific metal-trafficking pathways and metal ion chaperones has stemmed from studies in copper. However, it is highly likely that analogous pathways will exist for other metals as well. Metal ions such as iron, manganese, and even zinc are known to be toxic but essential nutrients. Therefore, it is critical that cells maintain these ions under strict guidance and regulation to ensure their proper incorporation into cognate metalloproteins while minimizing deleterious reactivity with other biological sites in the cell.

## See Also the Following Articles

Chaperones, Molecular • Cytochrome Oxidases, Bacterial

## Glossary

**ATX1** A copper chaperone for the copper-transporting P type ATPase in the Golgi.

**CCS** A copper chaperone for the largely cytosolic copper-zinc superoxide dismutase.

**copper COX17** A copper-carrying molecule for the mitochondria.

**metal chaperone** A molecule that binds a specific metal and helps insert this ion into the metal-binding site of a metalloprotein.

**metalloprotein** A protein that binds a specific metal ion and requires that metal ion for proper function.

**metal transporter** A transmembrane protein responsible for the translocation of metal ions across a lipid bilayer.

**SCO** A copper-carrying molecule, possibly the copper chaperone or copper insertion factor for cytochrome oxidase.

**transporting P type ATPase** A membrane transporter for copper that uses energy derived from ATP hydrolysis to drive copper transport.

## Further Reading

Culotta, V. C., and Gitlin, J. (2001). Disorders of copper transport. In *Molecular and Metabolic Basis of Inherited Disease*. (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, eds.) 8th edition, Vol. 2, pp. 3105–3126. McGraw-Hill, New York.

O'Halloran, T. V., and Culotta, V. C. (2000). Metallochaperones: An intracellular shuttle service for metal ions. *J. Biol. Chem.* **275**, 25057–25060.

Outten, C. E., and O'Halloran, T. V. (2001). Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **292**, 2488–2492.

Pufhal, R. A., Singer, C. P., Pearison, K. L., Lin, S. J., Schmidt, P. J., Culotta, V., Penner-Hahn, J. E., and O'Halloran, T. V. (1997). Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278**, 853–856.

Rae, T., Schmidt, P., Pufhal, B., Culotta, V. C., and O'Halloran, T. V. (1999). Undetectable intracellular free copper: The requirement of a copper chaperone for superoxide dismutase. *Science* **284**, 805–808.

## Biography

Valeria Culotta is a professor of environmental health sciences at the Johns Hopkins University Bloomberg School of Public Health in Baltimore, MD. She received her Ph.D. from the Johns Hopkins University Medical School and conducted her postdoctoral training at the National Institutes of Health. Her laboratory focuses on metal ion homeostasis and metal chaperones using baker's yeast as a genetic model system.

Edward Luk is a Ph.D. student in the Department of Biochemistry and Molecular Biology, Johns Hopkins University. His research interests are yeast genetics and metal ion biology.

# Chaperones, Molecular

**Sue Wickner and Joel R. Hoskins**
*National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA*

**A new concept in molecular biology that has evolved over the past 15 years is that proteins fold with assistance from other proteins, collectively referred to as molecular chaperones. All organisms, from bacteria to humans, possess several classes of molecular chaperones that are highly conserved throughout evolution. In general, molecular chaperones are proteins that bind to unfolded or misfolded proteins and facilitate protein remodeling without being part of the final complex themselves. Typically, they catalyze protein folding or unfolding in energy-dependent reactions. Chaperones participate in many cellular processes, including DNA replication, regulation of gene expression, protein synthesis, cell division, protection and recovery from stress conditions, membrane translocation, and protein degradation. There are four major classes of energy-dependent molecular chaperones: (1) GroEL or Hsp60, (2) DnaK or Hsp70, (3) Hsp90, and (4) Clp or Hsp100. All of these chaperones act in some situations in conjunction with cochaperones or accessory proteins. For example, DnaK acts with DnaJ/Hsp40 and GrpE, GroEL acts with GroES/Hsp10, Hsp90 associates with numerous partner proteins, and Clp proteins associate with proteases and other specificity factors to regulate protein degradation. This entry focuses on the role of Clp/Hsp100, Hsp90, and DnaK/Hsp70 chaperones in protein quality control. Another article in this encyclopedia will cover GroEL/Hsp60.**

## Protein Quality Control in the Cell

Molecular chaperones play a critical role in protein quality control during the course of cell growth as well as during stress conditions (Figure 1). Normal protein synthesis produces nascent unfolded proteins. Although some nascent polypeptides are able to fold spontaneously into their native conformation as they emerge from the ribosome (1 in Figure 1), others require the action of molecular chaperones, including members of the DnaK/Hsp70 and GroEL/Hsp60 families, to facilitate folding (2 in Figure 1). Unfolded and misfolded proteins also arise in cells as a result of environmental stresses, such as heat shock, or pathologic conditions, such as inflammation, tissue damage, infection, and genetic diseases involving mutant proteins. Molecular chaperones are able to refold and reactivate some misfolded proteins (2 in Figure 1). Other irreversibly misfolded proteins are recognized by proteases, such as Lon and Clp proteases in prokaryotes and the proteasome in eukaryotes. These multicomponent proteases use associated chaperones to unfold and deliver damaged proteins to the protease for degradation (3 in Figure 1). Finally, proteins that are neither refolded nor degraded form insoluble aggregates in the cell (4 in Figure 1). Aggregates are not always an end product, but can be dissolved by molecular chaperones. For example, the combined action of Clp/Hsp100 chaperones and DnaK/Hsp70 chaperones and cochaperones can carry out this function. Thus, chaperones play a critical role in protein quality control during both normal growth and cell stress by assisting proteins with functional potential to become properly folded and by ensuring that irreversibly damaged proteins are degraded.

## Clp/Hsp100 Chaperones

Clp ATPases, like the other chaperone families, are highly conserved and have been identified in many diverse organisms, including bacteria, archaea, yeast, plants, insects, and humans. They were first identified as molecular chaperones by S. Lindquist and colleagues and by S. Wickner and colleagues. Many organisms have multiple family members. For example, *Escherichia coli* has four Clp ATPases: ClpA, ClpB, ClpX, and HslU.

The Clp ATPase family is broadly divided into two classes. Class I Clp ATPases contain two nucleotide-binding domains, such as ClpA and ClpB in *E. coli*, Hsp104 in *Saccharomyces cerevisiae,*ClpC in plants, and ClpE in *Bacillus subtilis*. Class II Clp proteins contain one nucleotide-binding domain, such as ClpX in *E. coli*, yeast, plants, mice, and humans. Although the two ATP-binding domains of Class I differ from one another, they are highly conserved in all members of the class. The single ATP-binding domain of Class II Clp ATPases shares significant homology to the second ATP-binding domain of Class I. The Clp ATPases are members of the $AAA^+$ superfamily of ATPases (*A*TPases *a*ssociated with a variety of cellular *a*ctivities). The key feature of the superfamily is a

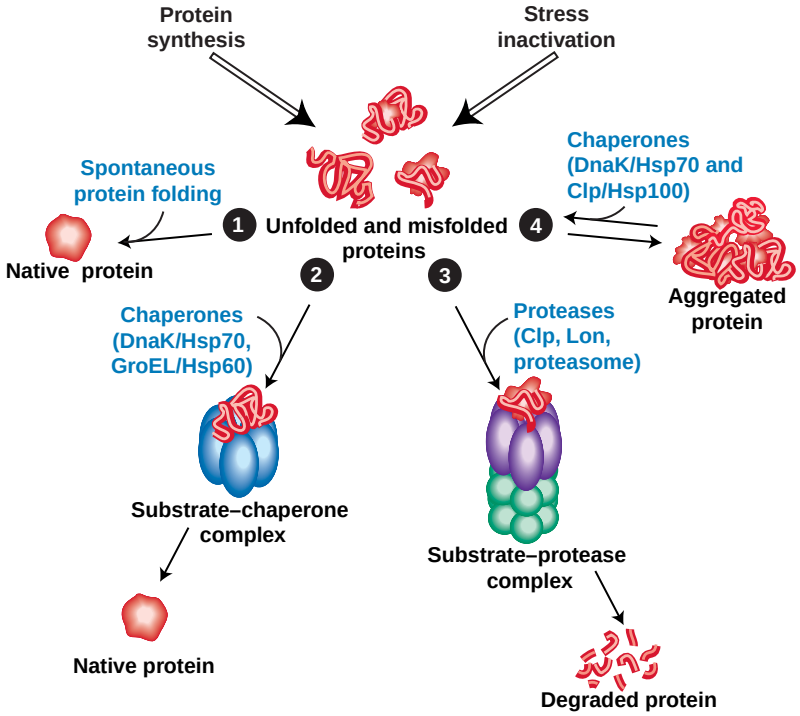



**FIGURE 1** Interplay of molecular chaperones and proteases in the cell. See text for description. Substrate proteins are shown in red; an ATP-dependent chaperone, such as GroEL, is shown in blue; a Clp chaperone is purple and is associated with a compartmentalized protease shown in green.

highly conserved ATP-binding module. Another feature is a protein recognition domain located C-terminally to the ATPase domain, referred to as the sensor-2 domain. Many $AAA^+$ ATPases, including many Clp ATPases, are components of molecular machines that function in a large number of diverse cellular processes.

Clp ATPases act as classical ATP-dependent chaperones. For example, ClpA performs ATP-dependent chaperone functions *in vitro* that mimic those of the DnaK/Hsp70 chaperone system. It catalyzes the remodeling of dimers of the plasmid P1 replication initiator protein into monomers. In an analogous reaction, ClpX dissociates the dimeric initiator protein of plasmid RK2 into monomers *in vitro*. ClpX participates in DNA transposition and replication of bacteriophage Mu by mediating the disassembly of MuA transposase tetramers from Mu DNA after recombination. Similar to other chaperones, both ClpA and ClpX prevent heat inactivation of proteins *in vitro*. Many of the Clp ATPases, such as ClpB, Hsp104, ClpX, and HslU, are induced by heat shock, suggesting that one of their *in vivo* roles is to prevent heat denaturation and to promote reactivation of heat-damaged proteins. Mutants in Hsp104 and ClpB are unable to grow at high temperatures, and *in vitro* Hsp104 and ClpB act in conjunction with their respective Hsp70 chaperone and cochaperones to resolubilize aggregates formed during exposure to high temperature or denaturants.

Some Clp chaperones associate with proteolytic partners, forming ATP-dependent proteases. The founding member of the Clp ATPase family, ClpA, was first identified as the ATPase component of a two-component ATP-dependent protease (referred to as ClpAP or protease Ti). ClpP, the proteolytic component, is unrelated to the Clp ATPases but is a member of a large family of serine proteases. By itself ClpP can degrade only short peptides, but when present as a complex with ClpA or ClpX it can degrade large proteins. Similarly, *E. coli* HslU associates with HslV, a proteolytic component, forming HslUV protease, and *B. subtilis* ClpE associates with ClpP of *B. subtilis*, forming ClpEP protease.

In view of the fact that Clp chaperones and proteases are generally not essential for growth, there is very likely an overlap in the functions carried out by the various cellular chaperones and proteases.

## Structure of Clp ATPases and Their Associated Proteases

The structures of the Clp ATPases that have been determined, including ClpA, ClpB, ClpX, and HslU, are very similar. Both electron microscopic and X-ray crystallographic studies show that Clp ATPases

self-assemble into oligomeric rings in the presence of ATP. The Clp ATPases that are known to be components of degradation machinery form stable complexes with their corresponding proteolytic component. The structure of one Clp ATPase, *Haemophilus influenzae* HslU, has been solved alone and in a complex with its proteolytic component, HslV, by McKay and colleagues (Figure 2). The HslUV structure shows that a hexameric ring of HslU ATPase binds to each axial end of the HslV proteolytic core. The proteolytic core is made up of two stacked rings of six identical subunits. The junction of the two stacked rings forms a chamber with the proteolytic active sites lining the interior chamber. With this molecular architecture, the active sites of the protease are sequestered from the cytoplasm. The structure of HslV is similar to other compartmentalized proteases, including the 20S proteolytic core of the proteasome and ClpP. The cavities formed by HslV, and also by ClpP, can accommodate unfolded proteins of roughly 30–40 kDa. Access to the proteolytic chamber appears to be through narrow pores at either end of the stacked rings; these small pores are only large enough to allow short polypeptides or unfolded proteins entry into the proteolytic chamber without major conformational changes. The pores of HslV are covered at one or both ends by the HslU ATPase rings, indicating the role of the Clp chaperone in regulating entry to the protease cavity. ClpAP and ClpXP have similar structures. The archaebacterial and eukaryotic 26S proteasomes are also similar to Clp proteases, in that regulatory ATPase components cover the pores of the 20S proteolytic core, indicating a common mechanism of action despite little sequence similarity between the analogous components.

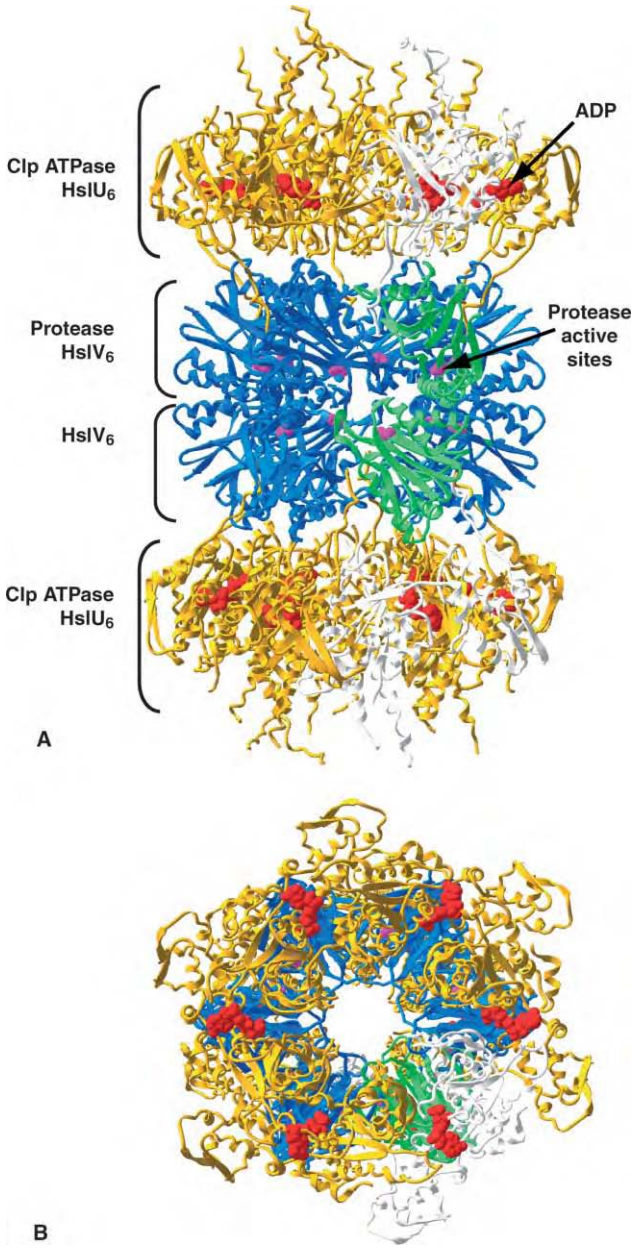



**FIGURE 2** Model of the crystal structure of a Clp ATPase, HslU, associated with a proteolytic component, HslV. (A) Side view. (B) Top view. HslU is represented in yellow, with one subunit per hexamer shown in white and ADP shown in red. HslV is represented in blue, with one subunit per hexamer shown in green and serine active sites shown in magenta. The crystal structure was solved by Sousa, M. C., Trame, C. B., Tsuruta, H., Wilbanks, S. M., Reddy, V. S., and McKay, D. B. (2000). Crystal and solution structures of an HslUV protease-chaperone complex. *Cell* **103**, 633–643.

## Mechanism of Action of Clp Chaperones and Proteases

The mechanism of action of Clp chaperones and Clp proteases is emerging from structural and biochemical data. Clp ATPases bind substrates that have Clp-specific recognition signals (1 in Figure 3). The recognition signals are approximately 10 amino acids and are typically found very near the N or C terminus of the protein. The various Clp ATPases recognize different motifs and thus act on separate but sometimes overlapping sets of substrates. As first shown by A. L. Horwich and colleagues, Clp ATPases catalyze ATP-dependent unfolding of bound substrates (2 in Figure 3). When Clp ATPases function as chaperones, they bind and unfold the substrate and then release the unfolded protein (3 in Figure 3). The released protein either refolds spontaneously (4 in Figure 3) or is rebound by Clp or another chaperone to undergo another cycle of unfolding. Multimeric complexes and aggregates can also be substrates for Clp chaperones. In these cases, the process of unfolding and release by the combined action of a Clp chaperone and the DnaK/Hsp70 chaperone system results in disassociation of the complex or aggregate.

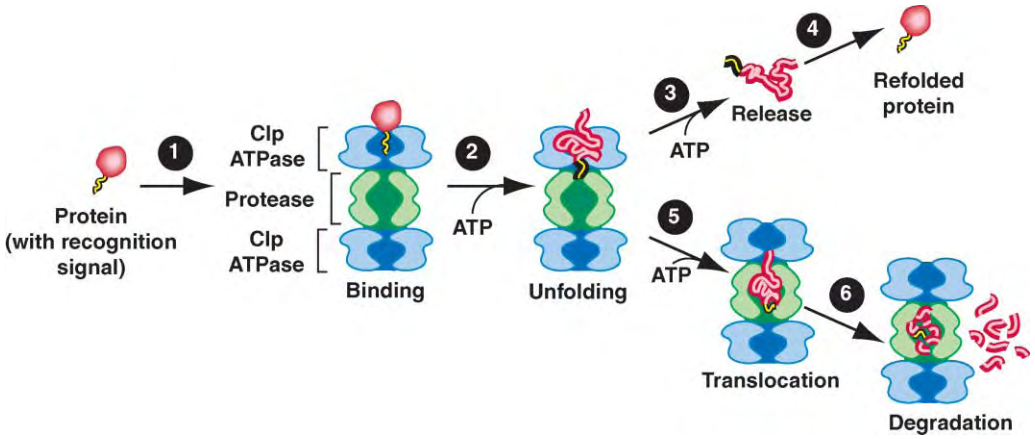



**FIGURE 3** Model of the mechanism of protein unfolding by Clp chaperones and degradation by Clp proteases. See text for description. A lateral cross section of a Clp chaperone is shown in blue, associated with a proteolytic core shown in green. A substrate protein is shown in red, with a recognition signal shown in yellow.

When Clp ATPases function as regulatory components of proteases, they specifically bind and unfold substrates (1 and 2 in Figure 3), but rather than releasing the unfolded substrate, the unfolded protein is translocated to the cavity of the protease in an ATP-dependent reaction (5 in Figure 3). The unfolded protein is threaded processively into the proteolytic cavity starting with the end containing the recognition signal. Degradation occurs in the proteolytic cavity (6 in Figure 3).

### Clp Specificity Factors or Cofactors

The activity of Clp ATPases is modulated in some situations by proteins that act as specificity factors. Specificity factors interact simultaneously with a substrate and a Clp ATPase. In this way they deliver otherwise unrecognizable substrates to a protease for degradation. There are examples of specificity factors that interact with ClpA, ClpX, and eukaryotic Clp homologues.

## DnaK/Hsp70

The most extensively studied DnaK/Hsp70 family member is the *E. coli* homologue DnaK. DnaK and its homologues consist of two domains, an amino-terminal ATPase domain and a carboxy-terminal peptide-binding domain. The structures of the ATPase domain and the peptide-binding domain have been solved separately (Figure 4). Because the structure of full-length DnaK has not yet been determined, it is still unknown how the two domains interact at the molecular level. Unlike GroEL and Clp chaperones, DnaK does not assemble into a multisubunit structure with a central cavity to accommodate substrates undergoing remodeling.

DnaK/Hsp70 chaperones act in conjunction with cochaperones. One cochaperone that is essential for DnaK/Hsp70 activity is DnaJ/Hsp40. DnaJ binds hydrophobic regions in substrate polypeptides and in addition interacts with DnaK. Another cochaperone is GrpE, a protein that stimulates nucleotide exchange by DnaK.

The current model for the mechanism of reactivation of heat-inactivated proteins by the DnaK chaperone system is that ATP-bound DnaK binds and releases heat-denatured polypeptides rapidly, through interactions with exposed hydrophobic region. DnaJ stimulates hydrolysis of ATP bound to DnaK, forming the ADP-bound state of DnaK, which stabilizes the DnaK–substrate interaction. GrpE binds to the ATPase domain of DnaK and induces nucleotide exchange. Conformational changes in DnaK accompanying ADP/ATP exchange force the release of the substrate and DnaJ. When released from DnaK, the substrate is likely in a partially unfolded conformation and either refolds spontaneously or is rebound by other chaperones or proteases.

## Hsp90 Chaperones

Hsp90 has been most extensively studied in eukaryotes, although there is a bacterial homologue, HtpG. The Hsp90 chaperone system is the most complex of the molecular chaperones in that it includes the Hsp70 chaperone system and a large number of cofactors. Hsp90 has an amino-terminal ATPase domain and a carboxy-terminal dimerization domain. The amino-terminal domain has been crystallized with

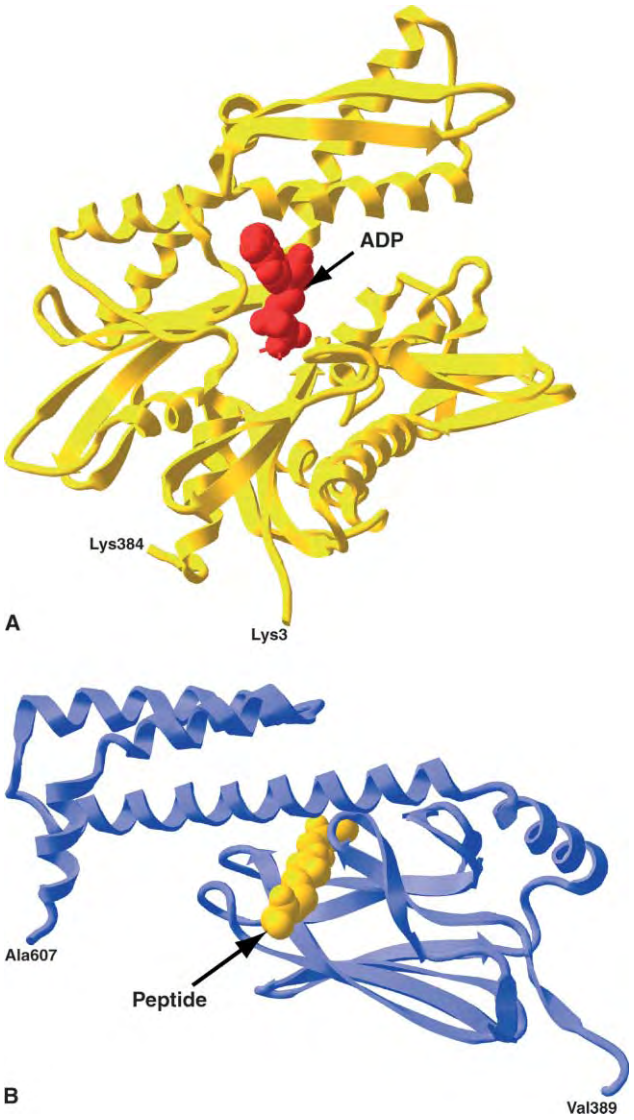



**FIGURE 4** Model of the crystal structure of a DnaK/Hsp70 chaperone. (A) Structure of the nucleotide-binding domain of a DnaK homologue, Hsc70, with bound ADP (shown in red). The structure was solved by Flaherty, K. M., Deluca-Flaherty, C., and McKay, D. B. (1990) *Nature* **346**, 623–628. (B) Structure of the peptide-binding domain of DnaK with a bound peptide (shown in yellow). The structure was solved by Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E., and Hendrickson, W. A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**, 1606–1614.

bound ATP (Figure 5). The mechanism of action of the Hsp90 system is less well characterized, but very likely it functions in a fashion similar to the other energy-dependent chaperones by remodeling substrate proteins through ATP-induced conformational changes. Hsp90 appears to be important for the folding of some proteins that are involved in key regulatory processes. It does not appear to play a major role in the de novo folding of proteins.

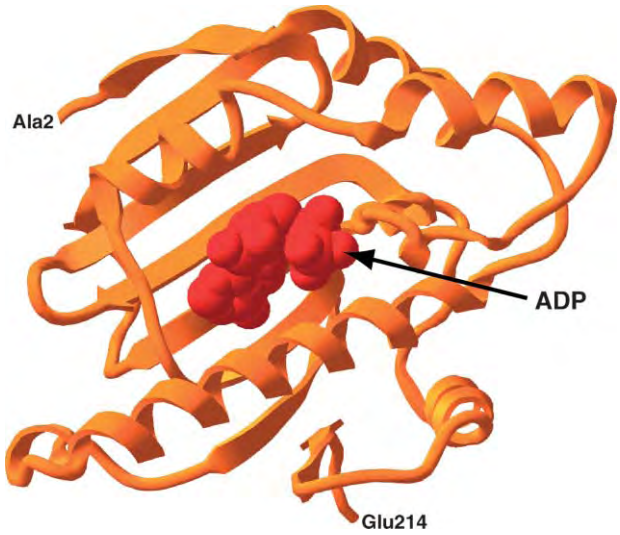



**FIGURE 5** Model of the crystal structure of the ATPase domain of Hsp90 with bound ADP (shown in red). The structure was solved by Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* **90**, 65–75.

## Summary

In summary, chaperones and proteases function together to determine the fate of proteins by facilitating the kinetic partitioning of substrates between pathways leading to reactivation, degradation, or, when quality control fails, aggregation.

## See Also the Following Articles

Chaperones for Metalloproteins • Chaperonins • Endoplasmic Reticulum-Associated Protein Degradation • Heat/Stress Responses • Protein Folding and Assembly • Unfolded Protein Responses

## Glossary

**AAA$^+$ ATPases** A large family of ATPases (AAA is derived from *A*TPases *a*ssociated with a variety of cellular *a*ctivities) characterized by a highly conserved 230–250 amino acid motif.

**Clp/Hsp100 chaperones** A large family of homologous ATPases that facilitate protein unfolding, some of which are induced by heat shock and are roughly 100 kDa in size.

**molecular chaperones** Specialized proteins that bind nonnative states of other proteins and assist them to reach a functional native conformation, in most cases through the expenditure of ATP.

## Further Reading

Bukau, B., and Horwich, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell* **92**, 351–366.

Dougan, D. A., Mogk, A., Zeth, K., Turgay, K., and Bukau, B. (2002). $AAA^{+}$proteins and substrate recognition, it all depends on their partner in crime. *FEBS Lett.* **529**, 6–10.

Fink, A. L. (1999). Chaperone-mediated protein folding. *Physiol. Rev.* **79**, 425–449.

Gottesman, S., Wickner, S., and Maurizi, M. (1997). Protein quality control: Triage by chaperones and proteases. *Genes Dev.* **11**, 815–823.

Horwich, A. L. (2001). *Advances in Protein Chemistry, Vol. 59. Academic Press, San Diego.*

Horwich, A. L., Weber-Ban, E. U., and Finley, D. (1999). Chaperone rings in protein folding and degradation. *Proc. Natl Acad. Sci. USA* **96**, 11033–11040.

Hoskins, J. R., Sharma, S., Sathyanarayana, B. K., and Wickner, S. (2001). Clp ATPases and their role in protein unfolding and degradation. *Adv. Protein Chem.* **59**, 413–429.

Patel, S., and Latterich, M. (1998). The AAA team: Related ATPases with diverse functions. *Trends Cell Biol.* **8**, 65–71.

Wickner, S., Maurizi, M. R., and Gottesman, S. (1999). Posttranslational quality control: Folding, refolding, and degrading proteins. *Science* **286**, 1888–1893.

## Biography

Sue Wickner is a Scientist in the Laboratory of Molecular Biology at the National Cancer Institute in Maryland. She received her Ph.D. from Albert Einstein College of Medicine and her postdoctoral training at the National Institutes of Health. Her principal research interests are in molecular chaperones and their role in proteolysis. Her more recent work helped to demonstrate that Clp ATPases are a class of ATP-dependent molecular chaperones and demonstrated that molecular chaperones act directly in protein degradation. She is a member of the National Academy of Sciences and the American Academy of Arts and Sciences and is a fellow of the American Association for the Advancement of Sciences.

Joel Hoskins received his B.S. from Catholic University and his M.S. from Johns Hopkins University. He is currently a Senior Research-Associate in the Laboratory of Molecular Biology in the NCI and has collaborated with Dr. Wickner on studies of molecular chaperones for 15 years.

# Chaperonins

**Arthur L. Horwich, Wayne A. Fenton and George W. Farr**
*Howard Hughes Medical Institute and Yale School of Medicine, New Haven, Connecticut, USA*

**Chaperonins are large ring assemblies that provide essential assistance in folding to the native state of a large variety of proteins through an ATP-driven mechanism. Substrate proteins acted on by these machines include many newly translated proteins in the cytosol of eubacteria, archaebacteria, and eukaryotes, and newly imported proteins inside eukaryotic mitochondria and chloroplasts. Chaperonins in many of these compartments are heat-shock proteins that help to restore native conformation under stress conditions. For example, in archaebacteria and eubacteria, a high basal abundance of ~1% of soluble protein can be increased by heat shock to more than 10%.**

## Two Classes of Chaperonin

Two classes of chaperonin have been distinguished, based on evolutionary and structural considerations (Table I). One class, termed type I, includes GroEL, Hsp60, and ribulose bis-phosphate carboxylase/oxygenase (rubisco)-binding protein. These reside in the bacterial cytoplasm and endosymbiotically related mitochondria and chloroplasts, respectively. Type I chaperonins function through cooperation with distinct co-chaperonins, e.g., GroES, Hsp10, and Cpn10 respectively. Co-chaperonins also form ring structures and dynamically associate coaxially with chaperonin rings, functioning as "lid" structures that encapsulate a protein substrate during folding in the central cavity. The other class of chaperonins, termed type II, includes TF55 (thermophilic factor 55) and the thermosome in archaebacteria and the eukaryotic cytosolic chaperonin, known as chaperonin containing TCP1 (CCT). This class differs architecturally from type I in containing a built-in lid structure.

## Overall Mechanism

Both classes of chaperonin function by the same general mechanism, a sequence of steps involving substrate protein binding, substrate folding in an encapsulated cavity, and release into the bulk solution (Figure 1). An open ring binds non-native protein in its central cavity through interactions between the cavity lining and exposed surfaces of the substrate protein, preventing misfolding and aggregation. In the case of type I chaperonins, this interaction occurs between the hydrophobic cavity lining of the chaperonin (Figure 2C) and exposed hydrophobic surfaces of the non-native protein, surfaces that will become buried to the interior of the substrate protein in the native state. Upon binding ATP and co-chaperonin in the case of type I, or ATP alone in the case of type II, large rigid body changes occur in the subunits of the ring, which dislocate its binding surface away from the central cavity, ejecting polypeptide into the cavity where it commences folding (Figure 1). Associated with the same movements, the cavity becomes encapsulated, in the case of type I chaperonins by binding co-chaperonin (Figure 2A) and in the case of type II chaperonins by the movement of protrusions comprising the built-in lid structure over the mouth of the cavity. Such encapsulation produces an environment in which polypeptide folds in isolation, prevented from forming any multimolecular interactions that could lead to aggregation. The enclosed chamber may also contribute to productive folding via a change in its wall character. In the case of type I chaperonins, there is a switch from the hydrophobic character of an open polypeptide-accepting ring to the hydrophilic character of the folding-active ring (Figure 2C). This switch presumably favors exposure in the folding polypeptide of hydrophilic surfaces and burial of hydrophobic ones, properties of the native state. Additionally, encapsulation in a confined space may also contribute to efficient folding, preventing population of a variety of extended off-pathway conformations.

Subsequent to the step of folding in an encapsulated space, shown to be the longest phase of the chaperonin reaction, hydrolysis of ATP permits release of the substrate protein from the central cavity into the bulk solution (Figure 1). For many substrate proteins, multiple rounds of binding to an open ring and folding inside the encapsulated cavity, followed by release into the bulk solution, are required to achieve the native state, with each round likely comprising an "all-or-none" trial at reaching the native state.

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Characteristics of Chaperonins**

| Chaperonin | Cellular compartment | Co-chaperonin | Subunits/ring | Subunit composition | Substrate proteins |
|---|---|---|---|---|---|
| *Type I* | | | | | |
| Hsp60 | Mitochondrial matrix | Hsp10 | 7 | Homo-oligomer | Many imported proteins |
| Rubisco binding protein | Chloroplast stroma | Cpn10 | 8 | $\alpha$, $\beta$ | Rubisco small subunit, other imported proteins |
| GroEL | Eubacterial cytoplasm | GroES | 7 | Homo-oligomer | Many cytoplasmic proteins, including $\lambda$ phage components |
| *Type II* | | | | | |
| TF55 | Archaebacterial cytoplasm | None | 9 | $\alpha$, $\beta$ | Unknown |
| Thermosome | Archaebacterial cytoplasm | None | 8 | $\alpha$, $\beta$ | Unknown |
| CCT (TRiC) | Eukaryotic cytosol | None | 8 | $\alpha$, $\varepsilon$, $\zeta$, $\beta$, $\gamma$, $\theta$, $\delta$, $\eta$ | Actin, tubulin, cyclin E, $G_\alpha$-transducin, VHL |

Those molecules failing, at the point of release into the bulk solution, to reach native form or a state that is committed to reaching native form in the bulk solution, are subject to an unfolding action upon rebinding to a chaperonin ring that likely returns them to the original non-native ensemble of states, enabling a fresh trial at folding.

The sum of these actions is the provision of kinetic assistance to the process of protein folding, effectively smoothing an energy landscape. That is, while the primary amino acid sequence of the substrate polypeptide provides all the information necessary to specifying a characteristic native conformation that typically lies at the energetic minimum, as articulated by Anfinsen and co-workers, during folding under physiologic conditions, there can be production of conformations that are kinetically trapped, preventing a protein from reaching the native state on a physiologic timescale. Chaperonins function in this context as biological catalysts that prevent or reverse formation of such misfolded species, thus enhancing the rate of folding *in vivo*.

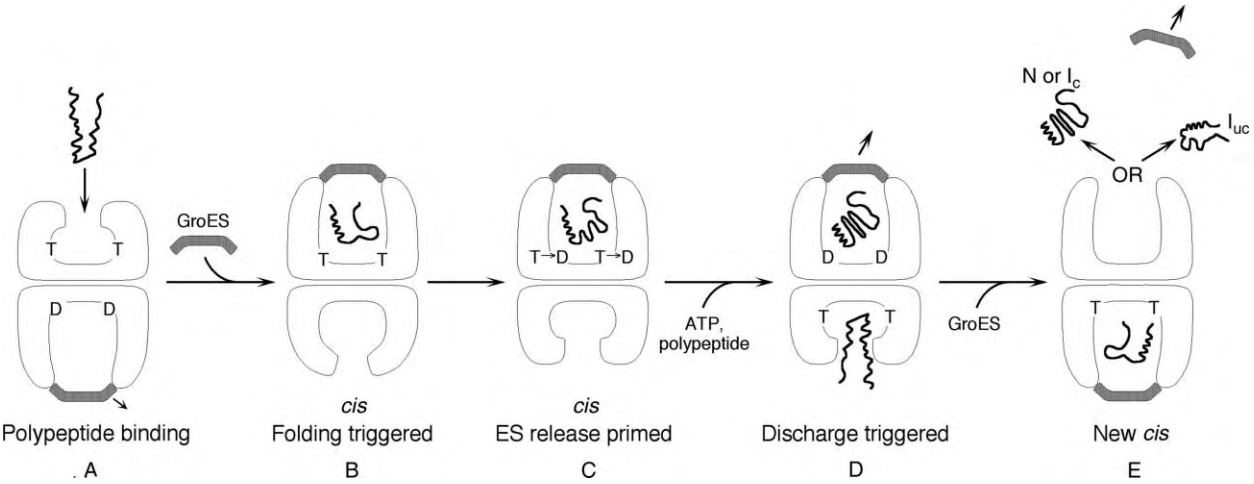



**FIGURE 1** The ATP-driven folding cycle of GroEL–GroES. Asymmetric GroEL–GroES–ADP complexes (A) are the most likely polypeptide-acceptor state *in vivo*. In the presence of ATP, GroES binds to the open ring, simultaneously discharging the substrate polypeptide from its binding sites and encapsulating it in the folding chamber (B). Folding proceeds in this sequestered environment, the longest step of the reaction cycle (~10 s), until ATP hydrolysis occurs (C). This weakens the GroES association and permits ATP and polypeptide to bind to the opposite ring (D). This discharges the ligands from the folding chamber and simultaneously establishes a new folding chamber in the opposite ring (E). Thus, the two rings of GroEL alternate asymmetrically between binding-active and folding-active states. The discharged polypeptides in (E) are in several possible states: N (native) or $I_c$, a state committed to becoming native without further chaperonin action; or $I_{uc}$, a non-native intermediate state that must rebind to chaperonin for another trial at folding. For many GroEL substrates, any given round of folding only produces a few percent of N or $I_c$ states; most of the population must be rebound for another attempt. In a cellular context, $I_{uc}$ states can also partition to other chaperone pathways or to degradative pathways. This latter option is vital for removing potential chaperonin substrates that are damaged or mutated such that they cannot fold.

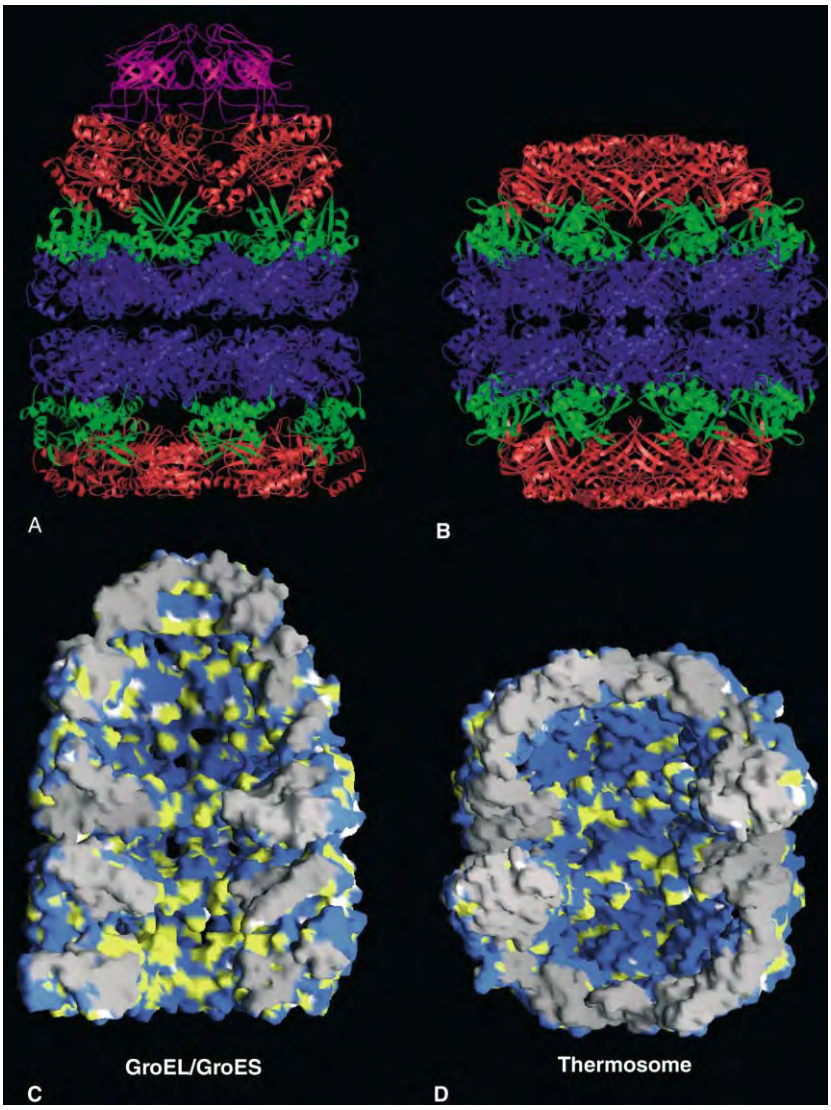



**FIGURE 2** Architecture of chaperonins. Structural models of a type I chaperonin, GroEL from *E. coli*, complexed with its co-chaperonin GroES (A, C) and structural models of a type II chaperonin, the thermosome from *T. acidophilum* (B, D). (A, B) Ribbon diagrams showing the domains of each chaperonin: equatorial, blue; intermediate, green; apical, red. In (A), GroES is colored magenta. (C, D) Space-filling models presented as cutaway views to show the interior cavities of the chaperonins, with surfaces colored according to hydrophobicity of the amino acid side chains: yellow, hydrophobic; blue, hydrophilic. Gray represents cutaway interface between subunits. Note the relative hydrophobicity of the lower (trans) ring of the GroEL/GroES, its polypeptide binding surface, as compared to the upper, GroES bound ring, relatively hydrophilic in character. Note also the absence of exposed hydrophobic surface in the type II complex.

## Architecture

Chaperonins are generally composed of two rings, stacked back-to-back, that function out of phase with respect to each other during the reaction cycle, such that only one ring is folding-active at a time. Each ring is composed of radially arranged identical or nearly identical subunits, each folded into three domains (Figure 2A, B). The "equatorial" domains, at the waistline of the cylinder, each contain an ATP-binding pocket. The collective of these domains comprises the stable base of the assembly, with the domains forming tight contacts with each other both side-by-side within a ring and across the ring–ring interface. The "apical" domains, at the end portion of the cylinder, are mobile, hinged at their bottom aspect, and contain the polypeptide-binding site at the inside surface. For type I chaperonins, this same binding surface, hydrophobic in character, is the one that associates with the co-chaperonin following rigid body elevation and twisting of the domains directed by ATP binding to the ring. These apical movements, captured by cryoEM and X-ray analyses of *E. coli* GroEL–GroES, amount to elevation by 60° and clockwise twist of 90°. They serve to dislocate the hydrophobic polypeptide-binding surface away from the central cavity, driving

polypeptide release into the cavity. Simultaneously, they enable GroES binding via interaction through its mobile loop segments, which extend down from each of the seven GroES subunits to contact the hydrophobic surface of each of the dislocated apical domains. This interaction is itself hydrophobic in character, mediated by an isoleucine-valine-leucine edge of the $\beta$-hairpin loop extended from GroES, which interacts with a portion of the hydrophobic binding surface of GroEL. This association of GroES with GroEL, and of other chaperonin–co-chaperonin pairs, results in encapsulation of the central cavity. For type II chaperonins, the apical domains each contain an insertion of residues, relative to type I, that comprises a protrusion, the collective of which function as a lid. The protrusions are marginated to the cavity wall of an open ring, but then are pointed centrally to collectively form a lid upon ATP binding. For both classes of chaperonin, the apical domains are covalently connected to the corresponding stable equatorial domains via a slender "intermediate" domain, which is hinged at both its top (apical) and bottom (equatorial) aspects (Figure 2A, B). This domain almost certainly contributes to transmitting the signal of ATP binding into the rigid body movements of the apical domains, and it also supplies a highly conserved aspartate residue that functions as a base in the mechanism of ATP hydrolysis: upon downward rotation of the intermediate domain attendant to ATP binding, the aspartate swings into the equatorial nucleotide pocket and activates water to attack the gamma phosphate of ATP.

The variations within different kingdoms on the basic theme of chaperonin architecture are interesting to contemplate and not fully understood (Table I). For example, an exception to the double-ring architecture is the mammalian mitochondrial chaperonin, which can be isolated as a single ring. Evidence has suggested that it may remain as a single ring throughout the nucleotide cycle, implying that it does not use the same allosteric ejection mechanism as the other chaperonins, which employ ATP binding on the opposite ring as a signal for release. The number of subunits per chaperonin ring also varies, with the bacterial and mitochondrial (type I) chaperonins containing seven subunits per ring, the chloroplast chaperonin containing eight subunits per ring, archaebacterial (type II) chaperonins containing eight or nine subunits per ring, and the eukaryotic cytosolic chaperonin (CCT) containing eight subunits per ring. Clearly, the volume of the central cavity, as well as the angle of adjacent subunits with respect to each other, is affected by the number of subunits per ring. Notably, because substrate proteins likely co-evolved with the machines that assist their folding, there could be stringency with respect to subunit number and ring size.

The hetero-oligomeric nature of some of the chaperonins also remains to be fully understood. For example, while GroEL and Hsp60 (type I) are homo-oligomers, the thermosome in archaebacteria (type II) contains eight-membered rings that are composed of $\alpha$–$\beta$ heterodimers in which the two subunits differ in amino acid sequence, principally in their apical domains. Most extreme is the eukaryotic cytosolic CCT chaperonin (type II), which contains eight different subunits per ring, arranged in a characteristic order. Here also, apical domain sequences are the most varied among the subunits (but any given subunit is well conserved across species lines, for example, from yeast to man). Studies of two major substrate proteins assisted by CCT, actin and tubulin, suggest that specific apical domains that are positioned at opposite aspects of the ring are involved in binding these proteins. Thus, it would seem that binding affinity for particular substrate proteins can comprise an evolutionary force directing apical domain evolution and hetero-oligomeric composition. Indeed, the overall character of the cavity wall of type I and II chaperonins seems to differ (Figure 2C, D). Rings of type I chaperonins present a hydrophobic cavity surface in the open state, with highly conserved hydrophobic residues positioned on a tier of three structures, two horizontally configured $\alpha$-helices and an underlying extended segment, that form the cavity-facing aspect of the apical domains (Figure 2C, bottom ring). By contrast, type II chaperonins present a surface, corresponding to these same secondary structures, that is not obviously hydrophobic – in fact, some of the residues that are hydrophobic and conserved in type I chaperonins are instead hydrophilic in type II (Figure 2D). Yet type II chaperonins harbor hydrophobic residues on their protrusions, and the potential role of these residues, as well as those lining the cavity wall, in substrate binding remains to be addressed.

## Polypeptide Binding by Type I Chaperonins

As mentioned, the step of polypeptide binding appears to be associated with capturing non-native states before they can irreversibly misfold and aggregate, and the step of binding, via exposed hydrophobic surfaces at least in the case of GroEL, appears to be associated with an action of unfolding. Such an action may be either a passive one, in which less folded states are preferred, with mass action shifting an ensemble of states in equilibrium with each other toward the less-folded ones, or unfolding may be catalyzed, where the polypeptide, bound by multiple apical domains simultaneously, is effectively unfolded. Either or both mechanisms could

be operative, but the net result for many canonical GroEL substrate proteins is a stably bound state that is weakly structured, e.g., very susceptible to exogenously added proteases and highly exchangeable in hydrogen/deuterium isotope exchange experiments. In the case of GroEL, crystallographic studies of peptides associated with the chaperonin apical domain have captured the peptides bound horizontally in the groove between the two $\alpha$-helices of the apical domain, forming hydrophobic contacts with those residues projected from the helices. Additional NMR studies reveal that other peptides are capable of forming $\alpha$-helices when they become associated with GroEL. Thus, it appears that at least some secondary structural elements can be preserved through the step of binding by chaperonin, but tertiary structure is unstable.

## The Nucleotide Cycle

The progression through the chaperonin reaction is driven by ATP binding and hydrolysis. This has been best studied in the case of the GroEL–GroES reaction (Figure 1). Here, ATP binds cooperatively to the seven equatorial sites within a ring but anti-cooperatively with respect to the opposite ring. Because GroES binding requires initial ATP occupancy of a ring, the GroEL–GroES complexes formed are asymmetric in character. The normal acceptor state for non-native polypeptide is an open ring of an asymmetric GroEL–GroES–ADP complex (Figure 1A). Once bound with polypeptide, a GroEL ring is converted to its folding-active state by the binding of ATP and GroES (Figure 1B). These produce the large rigid body domain movements associated with ejection of polypeptide into the central cavity and attendant folding. These steps can occur in the setting of a GroEL mutant unable to hydrolyze ATP and also in the presence of ADP–metal complexes such as ADP–beryllium fluoride (an ATP ground-state analogue) or ADP–aluminum fluoride (a transition-state analogue). In all of these cases, polypeptide proceeds to the native state inside a very stable GroES-encapsulated cavity. That is, the energy of ATP/GroES binding is sufficient to trigger the rigid body conformational changes that lead to ejection into the central cavity and productive folding. Thus, it is ATP binding, not hydrolysis, that is crucial for triggering folding.

The folding-active complex has a long half-life ($\sim$10 s for GroEL–GroES–ATP), after which ATP hydrolysis occurs (Figure 1C). This event does not interfere with folding but serves rather to weaken the stability of the GroEL–GroES complex. This "primes" the complex for discharge of its ligands, GroES, polypeptide, and ADP, effected by ATP binding to the opposite ring (Figure 1D). In sum, then, the GroEL–GroES machine functions asymmetrically, with only one ring folding-active at a time (and the other, opposite an ATP–GroES-occupied ring, empty of polypeptide and nucleotide until ATP hydrolysis occurs). The machine oscillates back and forth using one round of binding seven ATPs to simultaneously nucleate one folding-active ring and discharge the opposite ring that has completed its folding cycle (Figure 1E). It seems likely, given recent studies of CCT and archaebacterial chaperonins, that the same asymmetric cycle pertains there as well, with the apical protrusions mimicking the behavior of GroES with respect to the open or encapsulated state of a ring.

## Substrate Proteins

In general, small proteins (fewer than 100–150 amino acids) fold very rapidly and thus do not appear to require chaperonin assistance *in vivo*. Many larger proteins fold slowly, however, and are potential substrates for chaperonin-assisted folding. The actual *in vivo* substrates of chaperonins vary widely depending on chaperonin-type and subcellular compartment (Table I). GroEL in the bacterial cytoplasm is estimated to fold 10–30% of newly synthesized proteins, with particular preference for those between 20 and 60 kDa in size. Mitochondrial Hsp60 and the chloroplast rubisco-binding protein appear to be required for folding a potentially larger percentage of proteins newly translocated into these organelles. In the case of mitochondria, several large proteins, too large to be encapsulated inside the Hsp60 cavity, have been identified that require both Hsp60 and Hsp10. In the case of the yeast mitochondrial Krebs cycle enzyme, aconitase, studies with GroEL–GroES have resolved a *trans*-acting mechanism in which the polypeptide, unable to be encapsulated by GroES in *cis*, is productively released from the open ring by binding of ATP and GroES in *trans*.

The natural substrate specificity of archaebacterial (type II) chaperonins has not been defined, in part because these species have not been amenable to genetic manipulation. On the other hand, CCT, the type II chaperonin of the eukaryotic cytosol, may have a narrower substrate specificity than the other cytosolic chaperonins. Actin and tubulin are major substrates, yet additional cytosolic polypeptides have been shown to require CCT for efficient folding, including $G_\alpha$ transducin, cyclin E, and the von Hippel–Lindau tumor suppressor protein. For several of these substrates, evidence has emerged to suggest that they may be recognized in non-native form via exposed hydrophobic surfaces, as with type I chaperonins, yet the sites of binding on CCT itself remain to be identified.

## See Also the Following Articles

Chaperones for Metalloproteins • Chaperones, Molecular • Cytochrome Oxidases, Bacterial

## Glossary

**ATP** Adenosine triphosphate, the major energy metabolite in cells.

**cryoEM** An electron microscopy (EM) technique that involves rapid freezing of droplets of a protein solution to form a glass, which is then analyzed without further fixation or staining; as a result, the images closely represent the structural state of the protein in solution.

**energy landscape** The representation of the energetics of protein folding as a three-dimensional surface with hills and valleys (local energetic maxima and minima) to reflect the complexity of multiple interconnected pathways to the native state for the ensemble of non-native and intermediate folding states; this is in contrast to a two-dimensional reaction coordinate diagram used for simpler reactions.

**hydrophilic/hydrophobic** A description of the relative polarity of amino acid side chains in terms of their affinity for an aqueous environment. Hydrophilic side chains are polar, including ones with charged groups (e.g., lysine and aspartic acid) and ones with hydroxyl or amide groups (e.g., serine and asparagine). Hydrophobic side chains are non-polar, including aliphatic and aromatic groups (e.g., leucine and phenylalanine).

**rubisco** Ribulose bis-phosphate carboxylase/oxygenase, a major protein in the photosynthetic pathway.

## Further Reading

Cowan, N. J., and Lewis, S. A. (2001). Type II chaperonins, prefoldin, and the tubulin-specific chaperones. *Adv. Protein Chem.* **59**, 73–104.

Ellis, J. (ed.) (1996). *The Chaperonins*. Academic Press, San Diego.

Fenton, W. A., and Horwich, A. L. (2003). Chaperonin-mediated protein folding: Fate of substrate polypeptide. *Quar. Rev. Biophys.* **36**, 229–256.

Sigler, P. B., Xu, Z., Rye, H. S., Burston, S. G., Fenton, W. A., and Horwich, A. L. (1998). Structure and function in GroEL-mediated protein folding. *Annu. Rev. Biochem.* **67**, 581–608.

## Biography

Arthur Horwich is Professor of Genetics at Yale School of Medicine and an Investigator in the Howard Hughes Medical Institute. His research interests are in the general areas of cellular protein folding, unfolding, and misfolding. He holds an M.D. from Brown University and carried out postdoctoral research at the Salk Institute and Yale University. He is a member of the National Academy of Sciences.

Wayne Fenton is a Research Scientist in Genetics at Yale University School of Medicine. His general interests are in the mechanisms of protein folding and unfolding. He received a Ph.D. from Brandeis University and carried out postdoctoral work at Yale University.

George Farr is an Associate Research Scientist in the Howard Hughes Medical Institute. His research interest is in chaperonin-assisted protein folding. He received a Ph.D. from Case Western Reserve University and carried out postdoctoral work at Case Western and Yale University.

# Chemiluminescence and Bioluminescence

**Thomas O. Baldwin**
*University of Arizona, Tucson, Arizona, USA*

**Chemiluminescence and bioluminescence are terms that refer to the same physical process of light emission without heat. Bioluminescence is the process by which a living system or components isolated from a living system, such as a firefly tail, carries out a series of reactions that result in emission of light. Chemiluminescence is the same kind of process, but involving molecules that are not of biological origin. Chemiluminescence should be distinguished from incandescence, which occurs at high temperatures. Both bioluminescence and chemiluminescence are well known to the public, and both hold great fascination for all observers. Light without heat has intrigued people since the beginning of recorded history, and surely before.**

## Fluorescence: The Underlying Property

Fluorescence is a characteristic common to molecules formed as a result of chemiluminescent and bioluminescent reactions. At normal ambient temperatures, the vast majority of molecules exist in what is known as the ground state. However, under certain conditions, such as absorption of light energy, a molecule may be converted into an excited state. For fluorescent molecules, the excited state can return to the ground state by emitting energy as light. Virtually all molecules are fluorescent to some extent, but most emit such a small amount of light that sensitive instruments are required to detect it. A graphic description of fluorescence is shown in Figure 1.

In the case of fluorescence, the energy input is the absorption of light. The chemical characteristics of a molecule will determine which wavelengths (color) of light it will absorb. As in the ground state, the excited state will have multiple vibrational modes at discrete energy levels. At normal ambient temperatures, most of the molecules will be in the lowest vibrational energy level, so following absorption of the input energy, the molecules will relax to the lowest vibrational level of that excited state. Excited states tend to be very unstable, with lifetimes in the nanosecond to picosecond range. There are many ways for a molecule to return to the ground state. In solution, the excited molecules may collide with other molecules in solution and impart energy through the collisions, thereby increasing the vibrational and translational energies of the other molecules while itself returning to the ground state. Alternatively, some molecules, referred to as fluorescent molecules, have the ability to return to ground state by emission of light. Note that the emitted light will be of a lower energy (red-shifted in the spectrum) relative to that of the input energy due to the relaxation of the excited state. This effect is known as the Stokes shift.

## Chemiluminescence: Fluorescence from a Chemical Reaction

In the case of chemiluminescence, the input energy is derived from the making and breaking of bonds that occur during a chemical reaction. Other than that important detail, fluorescence and chemiluminescence result from the same fundamental characteristics of the molecules involved. For a reaction to be chemiluminescent, one of the products must be fluorescent, and the chemical step that results in generation of the fluorescent product must be of sufficiently high energy to result in formation of the excited state. Therefore, the efficiency of light emission from a chemiluminescent reaction is the product of the efficiencies of each step. That is, the chemiluminescent quantum yield, $\Phi_{CL}$, or the yield of photons per molecule of reactant consumed in the reaction, is given by the equation

$$\Phi_{CL} = \Phi_R \cdot \Phi_{CE} \cdot \Phi_F$$

All of these efficiencies have values between 0 and 1. The reaction yield, $\Phi_R$, is the chemical yield of the correct products, rather than the products of side reactions, which may not be fluorescent, and is typically near 1. The chemical excitation yield, $\Phi_{CE}$, is the fraction of the fluorescent product molecules that are produced in the excited state, rather than the ground state.

 © 2004, Elsevier Inc. All Rights Reserved. 

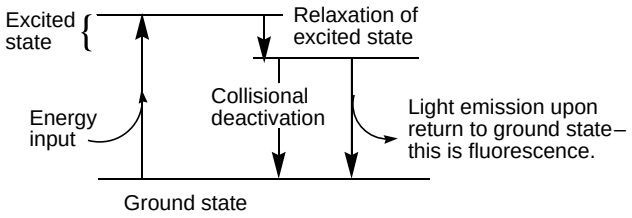



**FIGURE 1** Diagram showing the processes of generation of the excited state and return of the excited state to the ground state.

To be considered a chemiluminescent reaction, this value should be $10^{-3}$ or greater. Finally, the fluorescence quantum yield, $\Phi_F$, is the proportion of those molecules that are produced in the excited state that return to the ground state by emission of light rather than by collisional quenching or some other process, as depicted in Figure 1. The fluorescence quantum yield is typically 0.1 or greater. Conditions that impact any of these three factors will impact the overall yield of light from the reaction.

In some cases, product molecules are produced with exceptionally high chemical yield in the excited state, but the product is itself nonfluorescent (very low $\Phi_F$). In these cases, the yield of luminescence can be increased dramatically by addition of a highly fluorescent molecule to the reaction, which can, by a process called energy transfer, accept the energy from the excited product to become excited itself. The overall chemiluminescence quantum yield in such cases becomes

$$\Phi_{CL} = \Phi_R \cdot \Phi_{CE} \cdot \Phi_F \cdot \Phi_{ET},$$

where $\Phi_{ET}$ is the efficiency of energy transfer from the primary excited state produced in the chemical reaction to the acceptor, which then emits with the efficiency given by $\Phi_F$. The chemiluminescence quantum yields in some sensitized chemiluminescent reactions can be very high indeed, approaching 1.

Much research has been reported on the chemiluminescence properties of a wide array of peroxides, especially the cyclic peroxides such as the dioxetanes. Tetramethyldioxetane will decompose to yield 2 molecules of acetone, one in the singlet excited state and the other in the triplet (Figure 2). Of course, acetone is nonfluorescent, so the decomposition reaction is essentially dark. However, if 9,10-dibromoanthracene or 9,10-diphenylanthracene is added to the reaction, intense light emission is detected as a result of the energy transfer from the excited state acetone product to the fluorescent dye, DBA or DPA.

These and related technologies have found wide use in the form of chemiluminescence-based diagnostics and other applications. Perhaps one of the best known applications is the "light-stick" technology used by campers and hikers, and for fun and safety during Halloween. The light stick consists of a sealed, flexible plastic tube containing a solution of oxalic phthalate ester and a sealed glass vial containing a solution of hydrogen peroxide. Bending of the plastic tube will cause the glass vial to break, mixing the two solutions. The hydrogen peroxide will oxidize the ester, yielding 2 molecules of phenol and 1 molecule of 1,2-dioxetane-3,4-dione. The dioxetane will decompose to yield 2 molecules of carbon dioxide in the excited state. By including a dye sensitizer in the solution, the energy of the excited state carbon dioxide will result in light emission (see Figure 3). The color of the light emitted is determined by the fluorescence properties of the dye used in the light stick and can range from blue to red. Examples of dyes used are shown in Figure 3. One characteristic common to most fluorescent compounds and demonstrated in these examples is a system of conjugated double bonds.

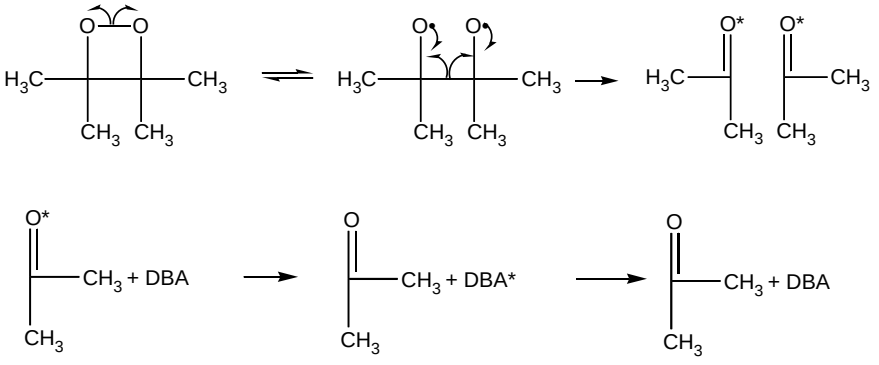



**FIGURE 2** Chemiluminescent decomposition of tetramethyldioxetane. The first step of the reaction is the hemolytic cleavage of the oxygen–oxygen bond, followed by hemolytic cleavage of the carbon–carbon bond to yield the diradical, which recombines to form acetone in the excited state. In the absence of the dye 9,10-dibromoanthracene, the reaction is dark. However, the dye can accept the energy from the excited state carbonyl to yield the excited state of the dye, which returns to ground state with the emission of light.

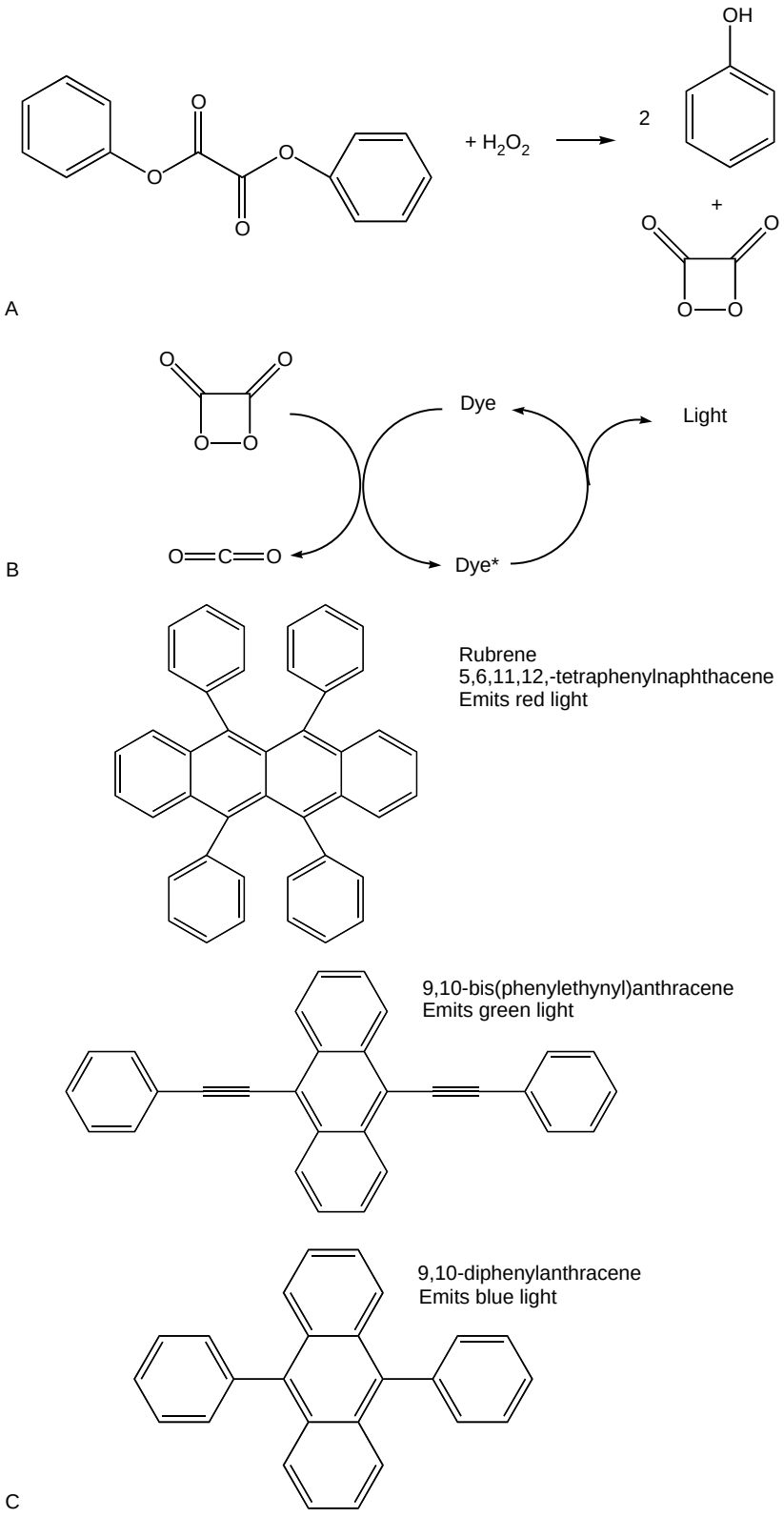



**FIGURE 3** The chemistry of the light stick. The reaction that occurs in a light stick upon breaking the glass ampule and allowing the two solutions to mix is shown in panel A. The oxalic phthalate ester is oxidized to yield 2 molecules of phenol and 1 molecule of the dioxetane. In panel B, the dioxetane decomposes by the same mechanism shown in Figure 2 to yield 2 molecules of carbon dioxide. The excited state carbon dioxide transfers energy to the dye to yield the excited state of the dye, which in turn emits light. The color of the light is determined by the chemistry of the dye. Three examples are shown in panel C, spanning the spectrum from red to blue.

## Bioluminescence: Chemiluminescence from a Biological Source

Bioluminescence reactions, unlike chemiluminescence, require an enzymatic catalyst for the reaction to occur. These enzymes are generically referred to as luciferases. Even though they have the same name, they catalyze vastly different reactions. For comparison, other groups of enzymes that have a common name, such as proteases, all catalyze the same kind of reactions. Proteases all hydrolyze peptide bonds, but luciferases catalyze reactions on completely different substrates, and they have no evolutionary relationship. Firefly luciferase, bacterial luciferase, and Renilla luciferase catalyze different reactions, having in common only the fact that light is emitted from a product of the reaction. It is therefore very important when discussing bioluminescence to stipulate the biological source of the enzyme.

There are many examples of bioluminescence that resemble the nonbiological chemiluminescence reactions described previously. For example, the reaction that occurs in the tail of the firefly is very similar to that of the light stick, but unlike the light stick, the firefly reaction requires the participation of an enzyme, firefly luciferase. As with chemiluminescent reactions, the firefly luciferase-catalyzed reaction has found numerous practical applications, primarily due to the involvement of ATP in the reaction (Figure 4). Many of these applications involve accurate and sensitive analysis of ATP levels within samples. The role of the ATP is to activate the carboxyl group of the firefly luciferin, the substrate for the luciferase-catalyzed reaction. As a result of this adenylation reaction, the luciferyl adenylate is now poised to react with molecular oxygen, eliminating AMP and forming the dioxetanone intermediate (Figure 5). The dioxetanone ring then decomposes by a mechanism similar to that shown in Figure 2, ultimately yielding $CO_2$ and the excited state of the product oxyluciferin, which emits light as it returns to ground state.

Different species of firefly emit light of different colors, but they all appear to use the same substrate luciferin, and there appear to be no energy transfer systems involved. At present, the detailed mechanism by which the insects emit light of different color is unknown, but it surely has to do with the details of the interactions of the excited state with the luciferase enzyme, since the enzymes are slightly different between the species. The color of the light emitted ranges from green to red, and the quantum yield of these reactions approaches 1.

Unlike firefly luciferase, which is a single polypeptide and employs ATP and a special substrate firefly luciferase, bacterial luciferase is a heterodimer ($\alpha\beta$) consisting of two similar but nonidentical polypeptides. In bacterial luciferase there is a single active center on the $\alpha$ subunit, but both subunits are required for the high quantum yield reaction. The substrates for the bacterial luciferase reaction are reduced flavin mononucleotide, molecular oxygen, and a long-chain saturated aldehyde. The enzyme formally is a flavin monooxygenase, catalyzing the cleavage of molecular oxygen and inserting 1 atom of oxygen into a substrate and the other into water. The mechanism of light emission in the bacterial luciferase-catalyzed reaction is still under debate, but there is general agreement regarding most of the reaction. In the first step of the reaction, reduced flavin mononucleotide ($FMNH_2$) reacts with $O_2$ to yield a reduced flavin peroxide (step 1, Figure 6). In the second step, the peroxide reacts with the aldehyde substrate to form the tetrahedral intermediate shown in Figure 6, step 2. There is general agreement in the field that this tetrahedral intermediate forms in the reaction, but how the intermediate decomposes to yield the excited state is under debate. One possible mechanism is shown in step 3 of Figure 6.

The identity of the emitter in the bacterial bioluminescence has been difficult to identify unambiguously because the product of the reaction, oxidized flavin mononucleotide, although fluorescent in solution, is nonfluorescent when bound to the luciferase. Various lines of evidence suggest that the emitter in the reaction is the flavin pseudobase shown in step 3 of Figure 6. The primary excited state, however, must be some other molecule, possibly the excited carbonyl shown in step 4 of Figure 6. In some species of bioluminescent bacteria, light emission from the living bacteria is significantly blue-shifted from that from the purified luciferase enzyme. It has been shown that the blue light comes from another protein, the lumazine protein, which becomes excited through some form of interaction with the luciferase during the reaction. This is an example of an energy transfer process in a bioluminescence

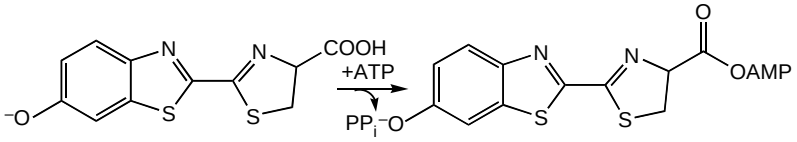



**FIGURE 4** Proposed mechanism of the firefly luciferase reaction. The luciferin reacts with ATP to form the luciferyl adenylate, thus preparing the molecule for reaction with oxygen.

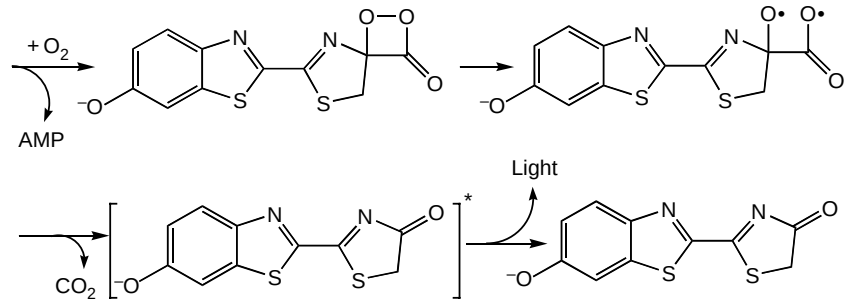



**FIGURE 5** Proposed mechanism of the firefly luciferase-catalyzed reaction. The luciferyl adenylate (Figure 4) reacts with molecular oxygen, eliminating the AMP and forming the cyclic peroxide dioxetanone structure. The dioxetanone then decomposes by homolytic cleavage of the O–O and C–C bonds, liberating $CO_2$ and yielding the excited state of the oxyluciferin. Light emission occurs as the excited state returns to ground state.

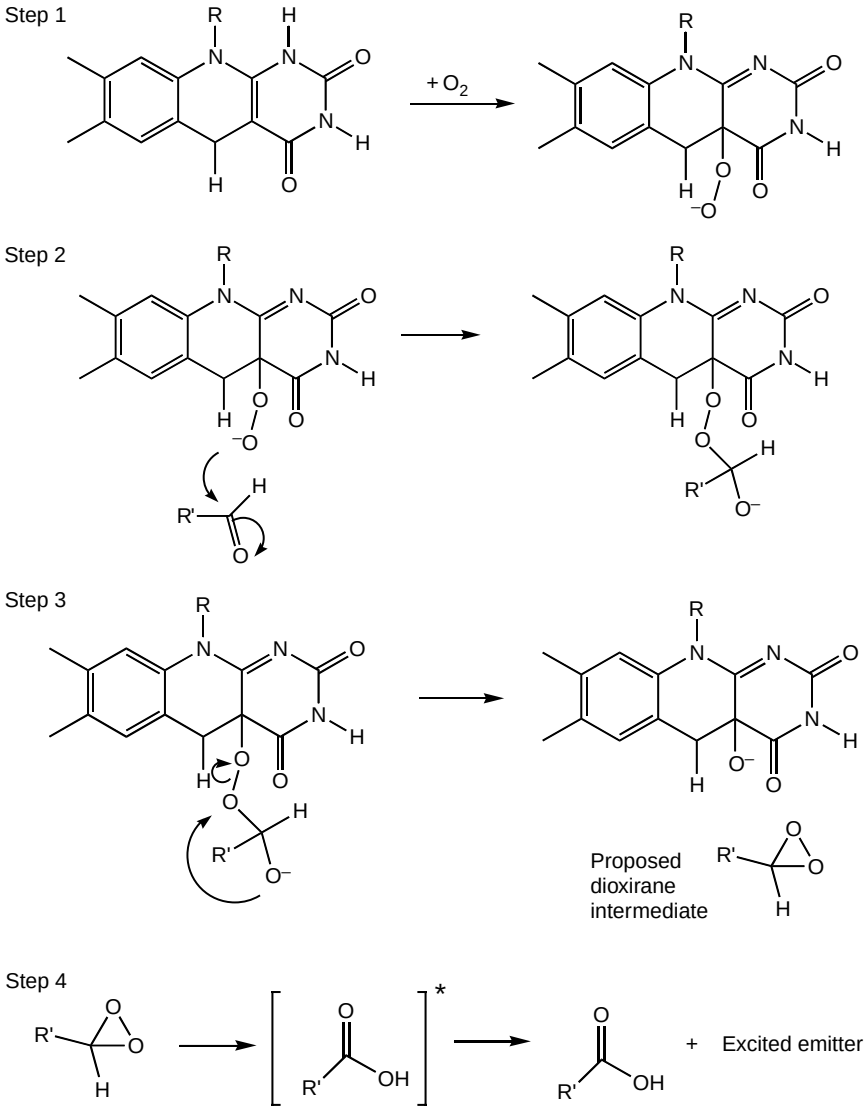



**FIGURE 6** Proposed mechanism of the bacterial luciferase-catalyzed reaction, as described in the text.

system, similar to the chemiluminescent examples described in Figure 3. But for the energy transfer to be efficient, it must occur in an energetically downhill direction. That is, the primary excited state must have an energy level higher than the excited flavin, which emits at a longer wavelength (lower energy) than does the lumazine protein. It is this fact, among others, that leads to the belief that the primary excited state in this reaction is likely to be an excited carbonyl, rather than the excited flavin. In the absence of the lumazine protein, energy transfer can occur to the flavin with emission from the excited flavin.

Another example of energy transfer in the bacterial bioluminescence system is from a yellow-emitting strain of *Vibrio fischeri*. These bacteria have a luciferase that emits blue-green light, similar to other bacterial luciferases, but *in vivo* and at temperatures of 20°C or lower, the color of the light emitted is bright yellow. This color shift is the result of a yellow fluorescence protein that has a highly fluorescent oxidized flavin mononucleotide chromophore. It is interesting that the lumazine protein, which causes a blue shift, and the yellow fluorescence protein appear to be homologous and carry out similar functions of energy transfer but do so with different chromophores.

There are many other examples of bioluminescent and chemiluminescent reactions, many of which have found important and valuable uses. The green fluorescent protein that was isolated from jellyfish is a very important tool in the study of protein trafficking and other molecular imaging applications. In immunoassays, chemiluminescent and bioluminescent reactions offer sensitivity, but without the hazards of radioactive labels. In recent years, work in the field has become primarily focused on applications of the technology; by comparison, very little effort is being directed at a basic understanding of the mechanisms of the processes involved. As should be apparent from this brief overview, much remains to be learned.

## See Also the Following Article

Flavins

## Glossary

**bioluminescence** The light that results from a chemiluminescent reaction in a living organism or from components of a living system, such as firefly tails.

**chemiluminescence** The light that is emitted from certain chemical reactions without the production of significant heat.

**flavin mononucleotide** Riboflavin-5′-phosphate, the 5′-phosphate derivative of vitamin $B_2$.

**flavin monooxygenase** An enzyme that employs a flavin coenzyme to split the two atoms of molecular oxygen apart, depositing one in an organic substrate as a hydroxyl group and the other in water.

**fluorescence** The phenomenon of light emission that occurs from certain molecules as they return to the ground state from the lowest excited singlet excited state. Population of the singlet excited state is the result of absorption of energy from incident light.

**luciferase** An enzyme that catalyzes a reaction that emits visible light with high efficiency. Luciferases are exceptionally diverse, many with no evolutionary relationship to the others.

## Further Reading

Baldwin, T. O. (1996). Firefly luciferase: The structure is known, but the mystery remains. *Structure* 4, 223–228.

Baldwin, T. O., and Ziegler, M. M. (1992). The biochemistry and molecular biology of bacterial bioluminescence. In *Chemistry and Biochemistry of Flavoenzymes*, Vol III, (F. Müller, ed.) pp. 467–530. CRC Press, Boca Raton, FL.

Harvey, E. N. (1952). *Bioluminescence*. Academic Press, New York.

Harvey, N. E. (1957). *A History of Luminescence from the Earliest Times to 1900*. American Philosophical Society, New York.

Lakowicz, J. R. (1999). *Principles of Fluorescence Spectroscopy*. Kluwer Academic/Plenum Publishers, New York.

Ziegler, M. M., and Baldwin, T. O. (eds.) (2000). *Bioluminescence and Chemiluminescence Part C. Methods in Enzymology*, Vol 305. Academic Press, New York.

## Biography

Thomas O. Baldwin received his graduate education at the University of Texas at Austin in the laboratory of Professor Austen Riggs. He then did postdoctoral studies at Harvard University, where he began his studies of the structure and function of bacterial luciferase. He has been on the faculty of biochemistry at the University of Illinois and Texas A&M University. In 1999, he moved to the University of Arizona, where he is professor and head of biochemistry and molecular biophysics and Founding Director of the Institute for Biomedical Science and Biotechnology.

# Chemiosmotic Theory

**Keith D. Garlid**
*Portland State University, Portland, Oregon, USA*

**Mitochondria transform the chemical energy derived from food and body stores into ATP by a process called oxidative phosphorylation. The chemiosmotic theory begins by describing the mechanism of coupling between substrate oxidation and phosphorylation. It goes on to describe the membrane properties that are required in order for mitochondria to provide ATP to the cell and, indeed, to survive within the cell. The chemiosmotic theory was presented as a hypothesis far in advance of experimental evidence, and it stands as a monument to the scientific method. For this extraordinary achievement, Peter Mitchell was awarded the Nobel prize in chemistry in 1978.**

## Basic Chemiosmotic Theory

### Mitochondrial Structure

Mitochondria are small, vesicular organelles. The internal aqueous compartment is called the *matrix*, which contains the enzymes of the Krebs tricarboxylic acid cycle. The matrix is enclosed by a highly folded, insulating membrane called the inner membrane, which contains the enzymic machinery of oxidative phosphorylation. The inner membrane is separated from the cytosol by a more permeable outer membrane, and the aqueous compartment between the inner and outer membranes is called the intermembrane space.

### The Four Postulates

Peter Mitchell proposed that nature uses protonic batteries to drive ATP synthesis and that biological energy conservation is essentially a problem in membrane transport, as diagrammed in Figure 1. The chemiosmotic theory consists of four postulates.

1. The inner membrane contains electron transport enzymes which are vectorially oriented so that the energy of electron transport drives ejection of protons outward across the membrane. The energy of substrate oxidation is thereby converted to and stored as a proton electrochemical potential gradient, called the protonmotive force.

2. The inner membrane contains a reversible, proton-translocating ATPase, which is also vectorially oriented so that the energy of ATP hydrolysis will drive protons outward across the inner membrane. The ATPase is reversible, so that protons driven inward through the enzyme by the redox-generated protonmotive force will cause ATP synthesis.

3. The inner membrane must have a low diffusive permeability to ions in general and to protons in particular. Otherwise, ion leaks would short-circuit the protonmotive batteries, and ATP would not be synthesized.

4. The inner membrane was postulated to contain exchange carriers in which anion entry is effectively coupled to proton entry. This provides a thermodynamically favorable pathway for substrate anions to reach enzymes within the electronegative matrix. The membrane was also postulated to contain exchange carriers in which cation exit is coupled to proton entry. This provides a thermodynamically favorable pathway for removal of cations that entered the matrix by diffusion down the very large electrical gradient caused by outward proton pumping.

## First Postulate – Respiration and the Electron Transport System

### Electron Transport

NADH and succinate arising from the tricarboxylic acid cycle are oxidized by the electron transport chain as diagrammed in Figure 2. The structure–function of the electron transport enzymes is discussed in other articles.

### Protonmotive Force

Electron transport through complexes I, III, and IV is coupled to electrogenic proton ejection across the inner membrane. The protonmotive force ($\Delta p$) is the free energy per mol required to move protons outward across the membrane. It is simply the sum of the work done against the electrical force and the work done against the

 © 2004, Elsevier Inc. All Rights Reserved. 

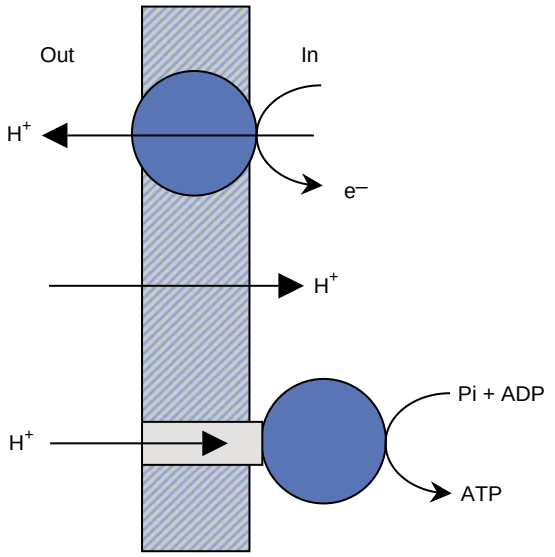



**FIGURE 1** Coupling of electron transport with ATP synthesis. The chemiosmotic theory states that vectorial enzymes of electron transport and ATP synthesis are coupled "indirectly" via the protonmotive force across the inner membrane of mitochondria. In order for oxidative phosphorylation to be coupled by this mechanism, the membrane must have a low permeability to protons (postulate 4).

proton concentration difference. $\Delta p$ is defined as the electrochemical proton gradient divided by the Faraday constant ($\Delta\mu_{H+}/F$); therefore

$$\Delta p = Z\Delta\text{pH} - \Delta\psi \qquad (1)$$

where $Z \equiv (RT\ \ln 10)/F = 59$ mV at 25 °C, and $\Delta\psi$ is the membrane potential (inside minus outside). $\Delta\psi$ and ΔpH can be estimated from equilibrium distributions of cationic dyes and weak acids, respectively. It is customary in bioenergetics to drop the negative signs of $\Delta\psi$ and $\Delta p$. Commonly observed values in isolated, nonphosphorylating mitochondria are 190 mV for $\Delta\psi$, 0.3 units for ΔpH, resulting in a $\Delta p$ of ~208 mV.

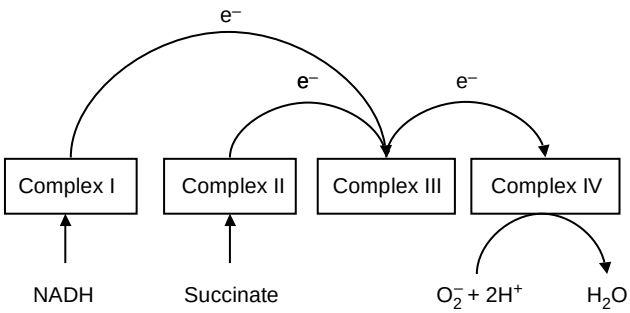



**FIGURE 2** Electron transport chain in mitochondria. Complex I (NADH-coenzyme Q reductase) and complex II (succinate coenzyme Q reductase) feed electrons into coenzyme Q at complex III (coenzyme Q cytochrome c reductase), then onto cytochrome c, which delivers them to complex IV (cytochrome *c* oxidase). At complex IV, molecular oxygen is reduced by the electrons to water. Electrogenic proton ejection occurs via complexes I, III, and IV.

## Stoichiometries

In the final step of electron transport, the dioxygen molecule ($O_2$) is reduced to water by four electrons ($e^-/O = 2$). When a pair of electrons moves from NADH to oxygen, it is estimated that ten protons are ejected across the inner membrane ($H^+/O = 10$).

## Respiratory Control

Respiration can readily be measured as oxygen uptake by isolated mitochondria. The typical traces in Figure 3 illustrate the principle of respiratory control, which is that respiration increases if the proton back-flux across the inner membrane is facilitated, either through the ATP synthase or by proton-translocating drugs or proteins.

## The Protonmotive Circuit

The chemiosmotic theory identifies the electron transport system (ETS) as a protonmotive cell, the behavior of which is identical to the well-known behavior of electromotive circuits, such as is shown in Figure 4. We note four salient aspects of this circuit: (1) The electron current is measured as respiration, as shown in Figure 3. (2) The current is determined entirely by the external resistances, and the battery will deliver increased current only when $R_e$ or $R_{ATP}$ are decreased.

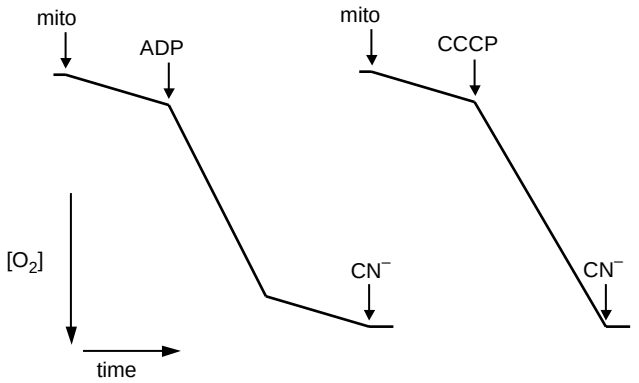



**FIGURE 3** Respiratory control in mitochondria. Mitochondria are added to a closed vessel containing an oxygen electrode and a medium containing phosphate and substrates to support respiration. A slow rate of oxygen consumption is observed, which is due primarily to proton leak across the inner membrane. When ADP is added, respiration accelerates until most of the ADP is consumed, after which it returns to the control rate. The protonophore CCCP accelerates respiration by catalyzing proton back-flux across the inner membrane. Respiration is stopped by addition of cyanide, which inhibits cytochrome *c* oxidase.

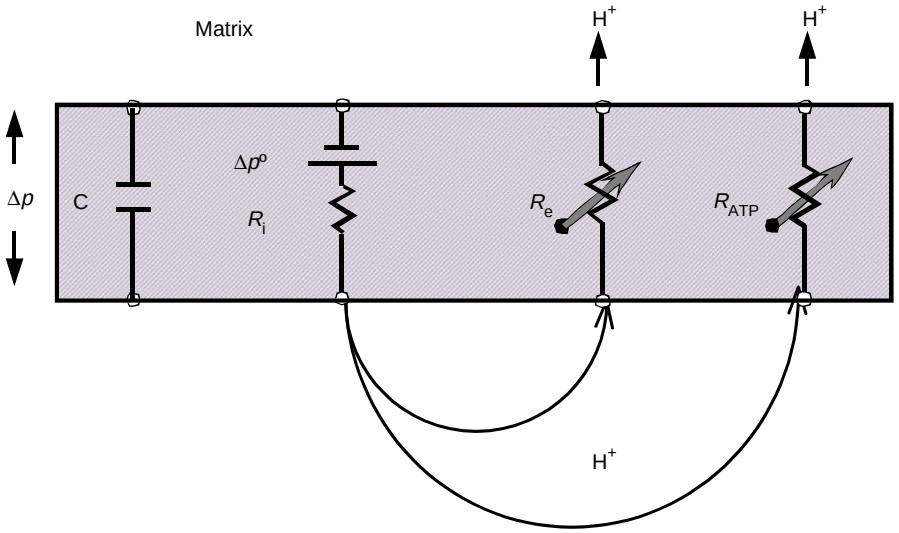



**FIGURE 4** Circuit diagram of the mitochondrial electron transport sytem (ETS). In the diagram, the battery symbol corresponds to the ETS, connected across the membrane through an internal resistance, $R_i$. C is the capacitance of the membrane. $\Delta p^o$ corresponds to the free energy drop when two electrons pass from the input redox couple to oxygen. $R_e$ is the sum of cation and proton leak resistances and those of the futile cation cycles necessary to regulate mitochondrial physiology. Proton back-flux through the ATP synthase is designated by the element containing $R_{ATP}$. If conductance through both of these back-flux pathways were zero, there would be no respiration, no proton pumping, and $\Delta p$ would equal $\Delta p^o$, the open-circuit voltage.

(3) The battery will respond the same whether current is drawn through $R_e$ or $R_{ATP}$. (4) As increasing current is drawn from the battery, the voltage will decrease, due to the internal resistance, $R_i$. Thus, respiration is "driven" by the free energy contained in the redox drop, and it is "controlled" by the proton back-flux through leak pathways and the ATP synthase.

## Behavior of the Protonmotive Circuit

The experiment in Figure 5 shows how $\Delta p$ varies when electron current is progressively increased by adding a protonophore that decreases external resistance ($R_e$) to $H^+$ ions. The resulting increase in respiration causes $\Delta p$ to fall gradually until the $V_{max}$ of the ETS is reached. What is being measured in such experiments is evident from the circuit diagram of Figure 4:

$$\Delta p = \Delta p^o - R_i \times V_O \qquad (2)$$

where $V_O$ is the respiration rate. The slope, $R_i$, is the internal resistance of the ETS, representing the weighted sum of frictional coefficients of the reactions leading to proton ejection. The intercept is $\Delta p^o$, the theoretical open-circuit voltage of the system, whose value is given by

$$\Delta p^o = (2/n_H)\Delta E \qquad (3)$$

where $n_H$ is the $H^+/O$ stoichiometry, and $\Delta E$ is the redox span being studied. The total redox potential, $\Delta E$, for a pair of electrons moving from NADH/$NAD^+$ to oxygen is ~1.16 V. Therefore, if $n_H$ is 10, $\Delta p^o = 232$ mV. Decreasing $R_e$ to increase proton back-flux may be achieved by ionophores, by uncoupling protein, or by futile $Ca^{2+}$ or $K^+$ cycling. Decreasing $R_{ATP}$ to increase proton back-flux may be achieved by adding ADP and

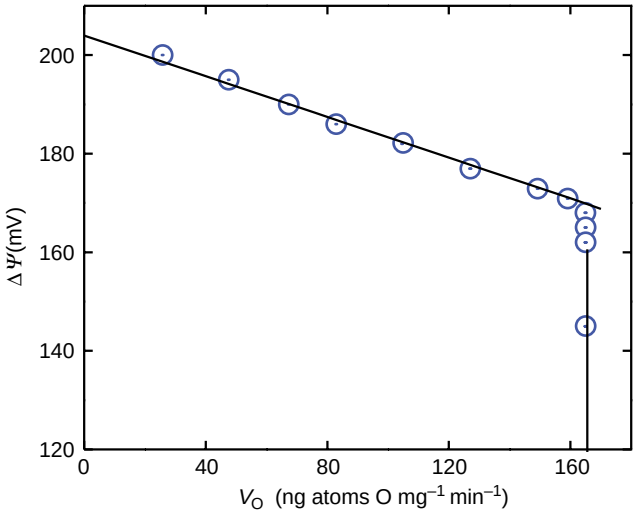



**FIGURE 5** Dependence of protonmotive force on electron transport rate. Membrane potential ($\Delta\psi$) of rat liver mitochondria is plotted versus respiration rate ($V_o$), which was varied by adding the protonophoretic uncoupler, CCCP. Respiration was measured using a standard Clark electrode. $\Delta\psi$ was determined from the distribution of tetraphenylphosphonium cation. The slope of the curve, $R_i$, is 0.35, the intercept is 210 mV, and $V_{max}$ is ~160 ng atom O $min^{-1}$ $mg^{-1}$. These are typical values for rat liver mitochondria respiring on succinate. The pH gradient was 0.3 and assumed to be invariant with increased respiration. Therefore, $\Delta p^o$ is ~228 mV.

phosphate so that current is drawn via the ATP synthase. Careful measurements show that all methods of increasing respiration yield points that fall on the same battery curve as illustrated in Figure 5.

## Second Postulate – The ATP Synthase

$\Delta p$ formed by the ETS is used to drive ATP synthesis via a remarkable series of steps. First, a proton binds to one of the 10–14 C subunits of the $F_0$ complex. This induces a conformational change that causes the C ring to rotate, after which the bound proton is released into a channel that carries it into the matrix. In this fashion, $\Delta p$ is transduced into a rotary mechanical force. The rotation of the C ring, in turn, drives the rotation of the attached $\gamma$-subunit, and rotation of $\gamma$ induces conformational changes in the catalytic sites of the three $\beta$-subunits of the $F_1$ head group of the ATP synthase. These catalytic sites exist in three different conformations, corresponding to the three faces presented by the end of the $\gamma$ subunit. If one proton is associated with each step of the cycle as it occurs on $F_1$, then the $H^+$/ATP stoichiometry would be 3 for ATP synthesis. On the other hand, if a complete revolution of the C ring is required for ATP synthesis, and if there are 12 C subunits in $F_0$, then the stoichiometry would be 4.

## Third Postulate – Ion Leaks and the Permeability Barrier

Notwithstanding the low diffusive permeability of the inner membrane, cation, and proton leaks occur at significant rates, and they are physiologically important. Inward $K^+$ leak causes matrix swelling, and inward proton leak contributes to the basal metabolic rate. Moreover, nature has engineered the uncoupling proteins to increase proton leak under certain physiological circumstances.

### Ion Leaks in Mitochondria

Diffusive transport of ions obeys the same laws that govern transport of nonelectrolytes across thin membranes. The rate is proportional to the concentration difference, and the proportionality constant (the permeability coefficient) is a function of the energy barrier that must be crossed during transport. The ionic charge adds a new complexity that derives from the long-range effects of the electric field on the local free energy of the diffusing ions. An ion diffusing across the inner membrane of mitochondria must cross an energy barrier whose maximum is located at the center of the membrane, and only those ions having sufficient energy to reach this peak will cross to the energy well on the opposite side. Net flux will therefore be proportional to the differential probability of getting to this peak from either side. This probability is given by the Boltzmann function, $\exp(-\Delta\mu_p/RT)$, where $\Delta\mu_p \equiv \mu_p - \mu_{aq}$ is the Gibbs energy of the ion at the peak ($p$) relative to its value in the aqueous energy well at the surface of the membrane ($aq$). These considerations lead to the following expression for diffusive flux of cations across thin biomembranes:

$$J = fxP(C_1 e^{u/2} - C_2 e^{-u/2}) \tag{4}$$

where u is the reduced voltage ($zF\Delta\psi/RT$), $C_1$ and $C_2$ are bulk aqueous concentrations, $f$ is the surface partition coefficient (energy well/bulk), and $P$ is the permeability constant, given by

$$P \equiv k e^{-\Delta\mu_p^{\circ}RT} \tag{5}$$

The factor 1/2 in the exponents of eqn. (4) arises from the fact that the maximum energy barrier is found at the midpoint of the membrane.

The second term in eqn. (4) represents back-flux of cations from the matrix and becomes negligible at the high values of $\Delta\psi$ maintained by mitochondria under physiological conditions. Thus, eqn. (4) reduces to a simple exponential function of $\Delta\psi$:

$$J = fPC_1 e^{u/2} \tag{6}$$

Eqn. (6) emphasizes the point that ion flux at high potentials is not affected by the concentration gradient across the membrane. Figure 6A contains data showing that proton leak is in good agreement with eqn. (6), and the flux–voltage plots in Figure 6B show that diffusion of $TEA^+$ (tetraethylammonium ion) and $H^+$ (hydronium ion) across the inner membrane behave identically with respect to their voltage-dependence.

### Uncoupling Proteins

Uncoupling proteins (UCPs) are the exception that proves the rule of the inner membrane permeability barrier. Nature devised the UCPs to intentionally short-circuit the inner membrane in order to dissipate energy and generate heat instead of ATP. UCP1 is expressed solely in brown adipose tissue, where it plays a major role in providing heat to hibernating animals and mammalian newborns. The human genome contains at least three additional UCPs, designated UCP2–4. UCP2 is ubiquitously expressed in mammalian tissues; UCP3 is expressed primarily in glycolytic skeletal muscle in humans; and UCP4 is expressed in brain. The roles of UCP in non-thermogenic tissue are uncertain but may involve reduction of mitochondrial production of reactive oxygen species.

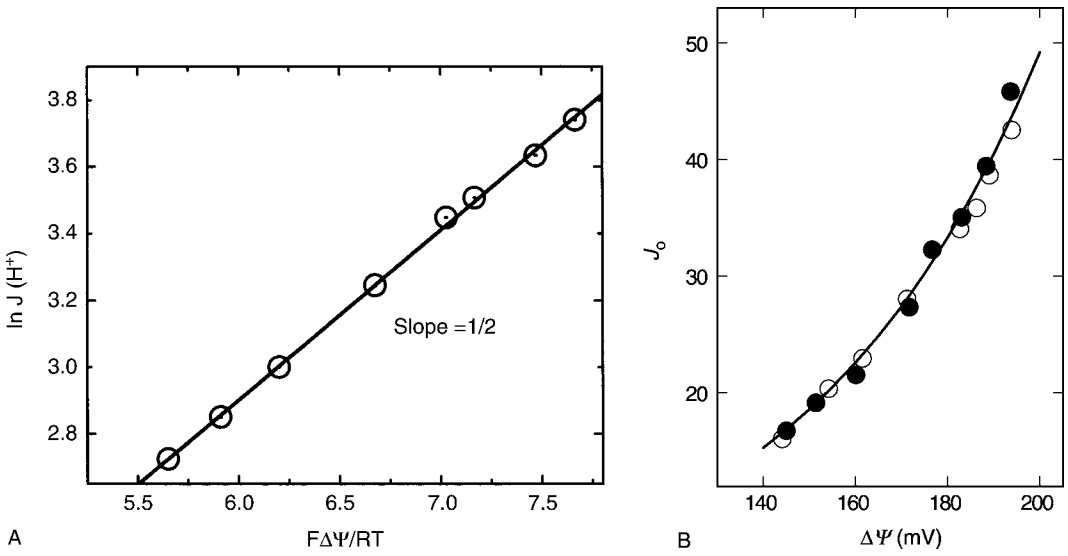



**FIGURE 6** Proton and cation diffusion across the inner membrane. (A) Proton flux. The semilogarithmic plot shows that $H^+$ leak is exponential with voltage, and the slope of ln $J_H$ vs. $F\Delta\psi/RT$ is 1/2, in agreement with eqn. (6). The intercept of such plots gives $J_o \equiv fP[C]_o$, where $[C]_o$ is the aqueous concentration, and $f$ and $P$ are defined in the text. (B) Tetraethylammonium ($TEA^+$) and $H^+$ flux. Fluxes were normalized to their respective values of $J_o$ and plotted vs. $\Delta\psi$. Note that the rate-limiting step of crossing the energy barrier is identical for $TEA^+$ and $H^+$ ($H^+$ probably crosses as hydronium ion).

The transport functions and regulation of UCP1–3 have been characterized using recombinant proteins expressed in *E. coli* and reconstituted into liposomes for flux measurements. The purified UCP proteins are qualitatively identical with respect to transport function and regulation. They catalyze electrophoretic flux of protons and alkylsulfonates, and proton flux exhibits an obligatory requirement for fatty acids. Fatty acid-dependent proton transport by UCP1–3 is inhibited by purine nucleotides, including ATP. The mechanism by which UCPs catalyze proton back-flux is somewhat controversial. It is thought that they act as fatty acid anion flipases, causing the outward movement of the fatty acid anion head group across the inner membrane. Once on the outer surface, the fatty acid picks up a proton and then flip-flops rapidly back into the matrix. Thus, UCP does not conduct protons, per se; rather, it enables fatty acids to behave as cycling protonophores, as shown in Figure 7.

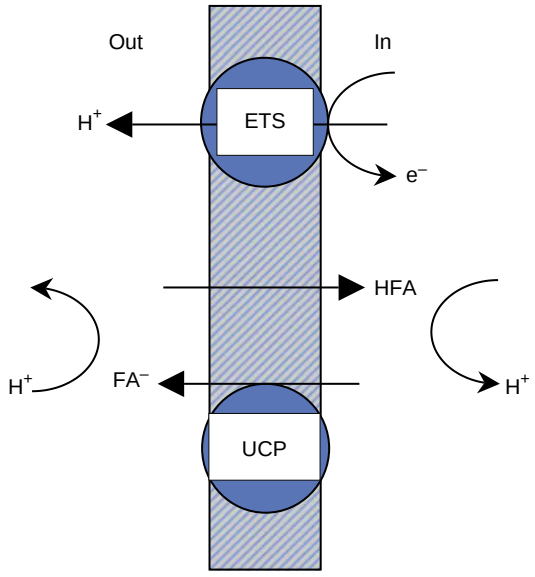



**FIGURE 7** Mitochondrial uncoupling protein (UCP). UCP contains a weak binding site for anions near the center of the membrane. This constitutes an energy well for anions and provides a low-resistance pathway for normally impermeant anions to cross the membrane. The physiological substrates of UCP are anions of free fatty acids (FA). The FA head groups are located at the acylglycerol linkages of the phospholipid bilayer. When the FA is at the surface of UCP, the negative membrane potential drives the head group to the energy well at the center of the membrane. The FA then "flip-flops" and the head group is driven to the opposite surface. Here, it diffuses away from the protein and picks up a proton. The protonated FA (HFA) head group freely diffuses across the membrane, during which the FA flip-flops again, and delivers a proton to the other side. Thus, UCP is a FA anion flipase, and its role is to enable FA to behave as cycling protonophores.

## Fourth Postulate – Ion Carriers and Channels

With astute physiological insight, Mitchell recognized that solving the problem of energy transduction gave rise to another. The high transmembrane electrical potential required for ATP synthesis would prevent anions, including the substrates of the tricarboxylic acid cycle, from entering the matrix. It would also promote cation uptake, with consequent osmotic swelling and

lysis. Thus, the fourth postulate was born out of physiological necessity.

## Anion Exchange Carriers

Because ATP is synthesized in the matrix, ADP and phosphate must be imported and ATP must be exported across the inner membrane. As shown in Figure 8, nucleotides are exchanged on the ATP/ADP translocase (ANT) in a process involving outward movement of one negative charge. The phosphate carrier catalyzes electroneutral $P_i/H^+$ symport or $P_i/OH^-$ antiport, with the net result that it transports phosphoric acid.

The inner membrane also contains a variety of anion exchange carriers, which are designed to deliver substrates to the tricarboxylic acid cycle. The anion exchange carriers catalyze 1:1 electroneutral exchange of anions, and they are arranged in a cascade in which phosphate and malate are key intermediates. In liver mitochondria, the dicarboxylic acid exchanger catalyzes malate/phosphate exchange, and the tricarboxylic acid exchanger catalyzes malate/citrate exchange. In this way, both di- and tricarboxylic acids are linked to the phosphate carrier. Since the phosphate carrier effectively transports fully protonated phosphate, the net result is that di- and tricarboxylic acids also behave as if they were fully protonated, and they are distributed across the membrane as if they were transported as fully protonated acids:

$$(A-)_{in}/(A-)_{out} = 10^{z\,\Delta pH} \qquad (7)$$

where $z$ is the valence of the acid.

## The Sodium–Calcium Cycle

The mitochondrial $Ca^{2+}$ cycle consists of three separate transporters and is diagrammed in Figure 9. $Ca^{2+}$ is taken up by the $Ca^{2+}$ channel at the expense of two ejected protons. In spite of the enormous gradient for electrophoretic $Ca^{2+}$ uptake, free mitochondrial [$Ca^{2+}$] is comparable to cytosolic [$Ca^{2+}$] *in vivo*. This disequilibrium is maintained in heart mitochondria by an electrophoretic $Na^+/Ca^{2+}$ antiporter, which exchanges 3 $Na^+$ per $Ca^{2+}$. The three $Na^+$ ions taken up are then ejected by the electroneutral $Na^+/H^+$ antiporter, which holds $Na^+$ close to equilibrium with the pH gradient.

The physiological role of the mitochondrial $Ca^{2+}$ cycle is to regulate matrix $Ca^{2+}$ activity in response to signals from the cytosol. As a second messenger, $Ca^{2+}$ signals need to increase cellular work. Because increased work requires a higher rate of ATP production, this

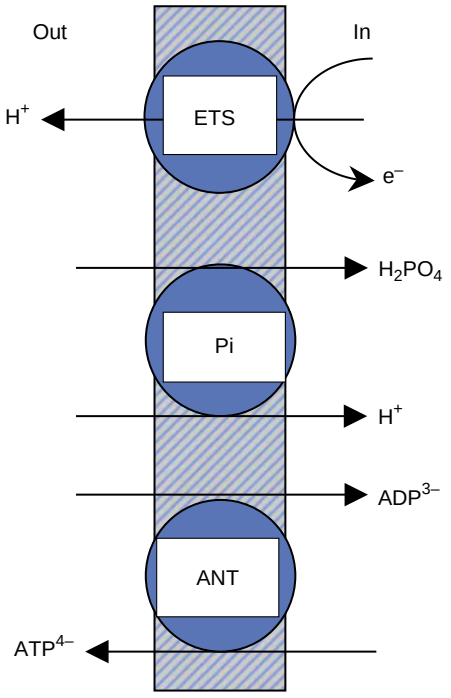



**FIGURE 8** Mitochondrial transport of inorganic phosphate, ADP, and ATP. The phosphate carrier (Pi) catalyzes electroneutral transport, and the adenine nucleotide translocase (ANT) catalyzes electrophoretic exchange of ADP and ATP. Uptake of Pi and ADP and expulsion of ATP use one electrogenically ejected proton.

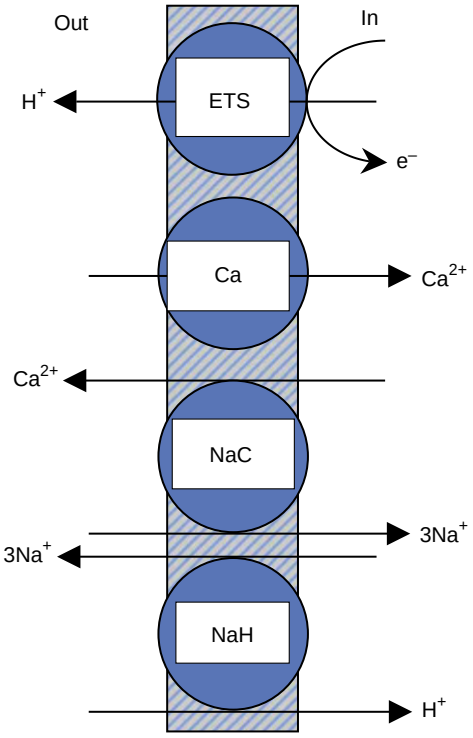



**FIGURE 9** The mitochondrial $Ca^{2+}$ cycle. $Ca^{2+}$ enters the matrix via the electrophoretic $Ca^{2+}$ channel and is ejected by the electrophoretic $Na^+/Ca^{2+}$ antiporter (NaC), utilizing three ejected protons per $Ca^{2+}$ taken up. The $Na^+$ is then expelled by the electroneutral $Na^+/H^+$ antiporter (NaH).

message must be relayed to the mitochondrial matrix. Intramitochondrial $Ca^{2+}$ is required to activate the phosphorylase that converts pyruvate dehydrogenase to its active form, and $\alpha$-ketoglutarate dehydrogenase is allosterically activated by matrix $Ca^{2+}$ in the physiological range.

## The Potassium Cycle

The mitochondrial $K^+$ cycle consists of electrophoretic $K^+$ influx and electroneutral $K^+$ efflux across the inner membrane, as diagrammed in Figure 10. Mitochondria must regulate net $K^+$ flux to zero in the steady state; otherwise, inward $K^+$ diffusion would cause the matrix to swell and eventually lyse. This regulation is provided by the $K^+/H^+$ antiporter, which ejects exactly the amount of $K^+$ that is taken in. Regulation of the $K^+/H^+$ antiporter is mediated by reversible binding of $Mg^{2+}$ and $H^+$ to the $K^+/H^+$ antiporter on its matrix side. The activity of these ions decreases with uptake of $K^+$ salts, causing a graded, compensatory activation of $K^+$ efflux in response to increases in matrix volume.

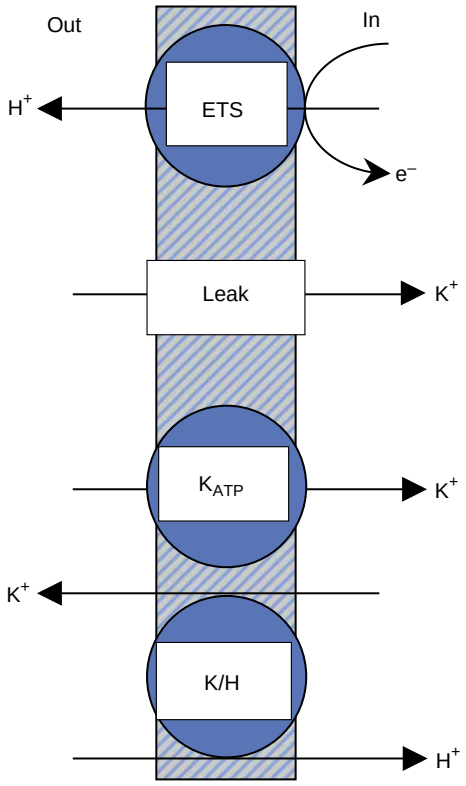



**FIGURE 10** The mitochondrial $K^+$ cycle. Electrogenic proton ejection drives $K^+$ uptake by diffusive leak. In addition, the inner membrane contains a KATP channel, which is highly regulated by nucleotides, CoA esters, and pharmacological agents. Net $K^+$ flux is regulated to zero in the steady state. Compensatory $K^+$ efflux is provided by the electroneutral $K^+/H^+$ antiporter, which is regulated by matrix $Mg^{2+}$ and $H^+$ and is exquisitely sensitive to changes in matrix volume.

The primary role of the $K^+/H^+$ antiporter is to provide "volume homeostasis" to mitochondria in order to maintain the vesicular integrity necessary for oxidative phosphorylation.

The mitochondrial $K_{ATP}$ channel (mito$K_{ATP}$) also plays an important role in volume homeostasis. When mito$K_{ATP}$ is open, the added $K^+$ conductance is thought to compensate for the lower driving force for $K^+$ influx (lower $\Delta\psi$) in ischemia and in high ATP-consuming states of the cell. Mito$K_{ATP}$ is regulated by a rich variety of metabolic and pharmacological ligands. It is inhibited with high affinity by ATP, long-chain acyl-CoA esters, the antidiabetic sulfonylurea, glyburide, and 5-hydroxydecanoate. The ATP-inhibited channel is opened with high affinity by guanine nucleotides and $K^+$ channel openers such as cromakalim and diazoxide. There is indirect evidence that mito$K_{ATP}$ is opened *in vivo* by phosphorylation. Mito$K_{ATP}$ has been found to play a pivotal role in protecting heart and brain from ischemic stress. Thus, opening mito$K_{ATP}$, either with $K_{ATP}$ channel openers or with endogenous signals, confers significant protection against ischemia–reperfusion injury.

## See Also the Following Articles

ATP Synthesis in Plant Mitochondria: Substrates, Inhibitors, Uncouplers • ATP Synthesis: Mitochondrial Cyanide-Resistant Terminal Oxidases • F1–F0 AT Synthase • Mitochondrial Channels • Mitochondrial Membranes, Structural Organization • Respiratory Chain and ATP Synthase • Tricarboxylic Acid Cycle • Uncoupling Proteins

## Glossary

**electrogenic transport** Ion transport (and net charge movement) that requires chemical energy to move an ion across the membrane against its electrochemical potential gradient. Electrogenic transport in mitochondria is limited to proton transport by the electron transport system and by the ATP synthase, when ATP is being hydrolyzed.

**electrophoretic transport** Ion transport (and net charge movement) driven by the ion electrochemical potential gradient. In mitochondria, this includes diffusion and transport by ion channels and ionophores such as valinomycin.

**ionophore** A chemical compound that conducts ions across membranes. Examples include valinomycin, CCCP, and nigericin.

**protonophore** An ionophore that conducts protons ($H^+$ ions) across membranes. Examples include dinitrophenol and CCCP (carbonyl cyanide *m*-chlorophenylhydrazone).

## Further Reading

Garlid, K. D., and Paucek, P. (2003). Mitochondrial potassium transport: The K+ cycle. *Biochim. Biophys. Acta* **1606**, 23–41.

Garlid, K. D., Beavis, A. D., and Ratkje, S. K. (1989). On the nature of ion leaks in energy-transducing membranes. *Biochim. Biophys. Acta* **976**, 109–120.

Garlid, K. D., Dos Santos, P., Xie, Z., Costa, A. D., and Paucek, P. (2003). Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K+ channel in cardiac function and cardioprotection. *Biochim. Biophys. Acta* **1606**, 1–21.

Hansford, R. G. (1994). Physiological role of mitochondrial $Ca^{2+}$ transport. *J. Bioenerg. Biomembr.* **26**, 495–508.

Mitchell, P. (1966). Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol. Rev. Camb. Philos. Soc.* **41**, 445–502.

Nicholls, D. G., and Ferguson, S. J. (2000). *Bioenergetics 3*. Academic Press, London.

## Biography

Keith D. Garlid is a Professor of Biology at Portland State University, in Portland, Oregon. He holds an M.D. from Johns Hopkins University, and a Doctor tecnicae degree from the Norwegian Institute of Technology. His principal research interests are in mitochondrial physiology and bioenergetics. He has published extensively on the mitochondrial uncoupling proteins and on the mitochondrial potassium cycle. His paper showing that the mitochondrial ATP-sensitive $K^+$ channel is the receptor for drugs that protect the heart from ischemia–reperfusion injury has had a major impact on ischemia research.

# Chemokine Receptors

**Ann Richmond and Guo-Huang Fan**
*Department of Veterans Affairs, VA Medical Center, Nashville, Tennessee, USA and Vanderbilt University School of Medicine, Nashville, Tennessee, USA*

**Chemokine receptors are seven-transmembrane proteins expressed on neutrophils, lymphocytes, dendritic cells, and many other cell types. They function to mediate migration of leukocytes to the sites of injury and infection in response to a gradient concentration of chemokines, a group of small proteins that specifically bind the chemokine receptors. Chemokine receptors belong to the G protein-coupled seven-transmembrane receptor superfamily, which has more than 600 members that use the heterotrimeric G proteins to initiate signals. Eighteen chemokine receptors have been identified and cloned so far, and these receptors have been classified into four subfamilies (CXC, CC, CX3C, and C) based on the chemokine subclass specificity. In addition to the mediation of migration of leukocytes to the inflammatory sites (chemotaxis), chemokine receptors play a role in leukocyte homing, HIV entry, angiogenesis, tumor growth and metastasis, development, and inflammation of the central nervous system.**

## Ligands

The ligands that bind and activate chemokine receptors are chemokines or chemoattractant cytokines. Approximately 50 chemokines have been identified so far, and these chemokines are classified into four (CXC, CC, C, and CX3C) subfamilies based on their primary amino acid sequences (Table I). The CXC subfamily has six members, which have one amino acid (X) interrupting the first two of their four conserved cysteine residues. The CC subfamily has 10 members, which have no intervening amino acid between the first two of their four cysteine residues. In both the CXC and CC subfamilies, disulfide bonds are formed between the first and third cysteines and between the second and fourth cysteines to establish a stable tertiary structure with the molecular mass of 7–9 kDa. The C subfamily has two members, which are 16 kDa in molecular size. The CX3C subfamily has only one member (CX3L1), which has three intervening amino acids between the N-terminal cysteines, with a molecular mass of 38 kDa, larger than any other known chemokine. Chemokines have two main sites of interaction with their receptors, the flexible N-terminal region and the conformationally rigid loop that follows the second cysteine. Chemokines dock onto receptors by means of the loop region, and this contact is necessary to facilitate the binding of the N-terminal region to the receptor that results in receptor activation. Chemokines possess heparin-binding capacity at their C-terminal end, which enables them to bind to glycosaminoglycans and other negatively charged sugar moieties on cell surfaces and matrix glycoproteins. This property may result in the adsorption of chemokines onto the endothelial cell lining of the blood vessels, connective tissues, and cell matrices. Thus, chemokines immobilized on tissue or matrix surfaces may induce haptotactic migration of target cells.

## Structure

The chemokine receptors generally are composed of 340–370 amino acid residues and have 25–80% identity. These receptors share a common putative structural topology composed of seven hydrophobic transmembrane domains, an N terminus outside the cell surface, three extracellular and three intracellular loops, and a C terminus in the cytoplasmic compartment. The poorly conserved N-terminal domains together with a second binding site in the extracellular loops determine the specificity for ligand binding. The sequence DRYLAIVHA, or a variation of it, in the second intracellular loop and the third intracellular loop is required for G protein coupling. A cysteine residue in each of the four extracellular domains is required for the disulfide-bond formation that is critical for cell surface expression. The C terminus of the receptors contains a number of serine and threonine residues that, upon phosphorylation, are involved in signaling and receptor desensitization. A leucine–leucine or isoleucine–leucine motif in the C terminus is required for the receptor internalization (Figure 1). Some chemokine receptors such as CCR2, CCR5, and CXCR4 form homodimers, which may be needed for signal transduction.

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Chemokine Receptor Family**[a]

| Name | Main agonists | Main functions |
|---|---|---|
| CXCR1 | CXCL8 | Neutrophil migration; innate immunity; acute inflammation |
| CXCR2 | CXCL1–3, CXCL5–8 | Neutrophil migration; innate immunity; acute inflammation; angiogenesis |
| CXCR3 | CXCL9–11 | T-cell migration; adaptive immunity; Th1 inflammation |
| CXCR4 | CXCL12 | B-cell lymphopoiesis; bone marrow myelopoiesis; central nervous system and vascular development; HIV infection |
| CXCR5 | CXCL13 | B-cell trafficking; lymphoid development |
| CXCR6 | CXCL16 | T-cell migration |
| CCR1 | CCL3, CCL5, CCL7, CCL8, CCL13–16, CCL23 | T-cell and monocyte migration; innate and adaptive immunity; inflammation |
| CCR2 | CCL2, CCL7, CCL8, CCL13 | T-cell and monocyte migration; innate and adaptive immunity; Th1 inflammation |
| CCR3 | CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26 | Eosinophil, basophil, and T-cell migration; allergic inflammation |
| CCR4 | CCL17, CCL22 | T-cell and monocyte migration; allergic inflammation |
| CCR5 | CCL3, CCL4, CCL5, CCL8, CCL14 | T-cell and monocyte migration; innate and adaptive immunity; HIV infection |
| CCR6 | CCL20 | Dendritic cell migration |
| CCR7 | CCL19, CCL21 | T-cell and dendritic cell migration; lymphoid development; primary immune response |
| CCR8 | CCL1, CCL4, CCL17 | T-cell trafficking |
| CCR9 | CCL25 | T-cell homing to gut |
| CCR10 | CCL26–28 | T-cell homing to skin |
| CX3CR1 | CX3CL1 | T-cell and NK cell trafficking and adhesion; innate and adaptive immunity; Th1 inflammation |
| XCR1 | XCL1–2 | T-cell trafficking |

[a] Modified from Murphy, P. M., Baggiolini, M., Charo, I. F., Hébert, C. A., Horuk, R., Matsushima, K., Miller, L. H., Oppenheim, J. J., and Powe, C. A. (2000). International Union of Pharmacology, XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev.* **52**, 145–176, National Institutes of Health.

## Consequence of Receptor–Ligand Interaction

### Signal Transduction

Ligand binding to chemokine receptors initiates a cascade of intracellular events (Figure 2). The first step is the conformational change of the chemokine receptors, inducing the exchange in the $\alpha$-subunit of the G proteins from the GDP to GTP bound state dissociating the $\alpha$ from the $\beta$ and $\gamma$ G protein subunits. The G$\beta\gamma$ subunits activate phospholipase C $\beta$2 (PLC$\beta$2), which hydrolyzes phosphatidylinositol 4,5-bisphosphate [(4,5)P2] to produce inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 mediates $Ca^{2+}$ release from the intracellular stores. DAG activates protein kinase C (PKC), which phosphorylates a number of effector molecules. The activation of isotypes of PLC and hydrolysis of PIP2 vary among chemokine receptors and from cell type to cell type, which may account for the divergent cellular responses to a given chemokine. Another important signaling pathway of chemokine receptors is the activation of phosphoinositide 3-kinases (PI3K), which convert the plasma membrane lipid PI (4,5)P2 to phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3], resulting in the phosphorylation and activation of the serine-threonine kinase Akt (PKB). An additional part of the signals that evoke the motility response involves activation of the rho GTPase family including rho, rac, and cdc42, which can feed into the activation of $P^{21}$ activated Kinase (PAK), Wiskott-Aldrich syndrome protein (WASP), and Arp2/3 to mediate actin cytoskeletal changes. Chemokines also induce tyrosine phosphorylation of p130 Cas, focal adhesion Kinase (FAK), and members of the src family in several cell types. Through activation of the ras, raf, MAPK cascade and through activation of the NF-$\kappa$B cascade, transcription factors are activated and gene expression is affected. The signaling pathways of chemokine receptors play a role in cell growth, cell migration, cell survival, and superoxide production.

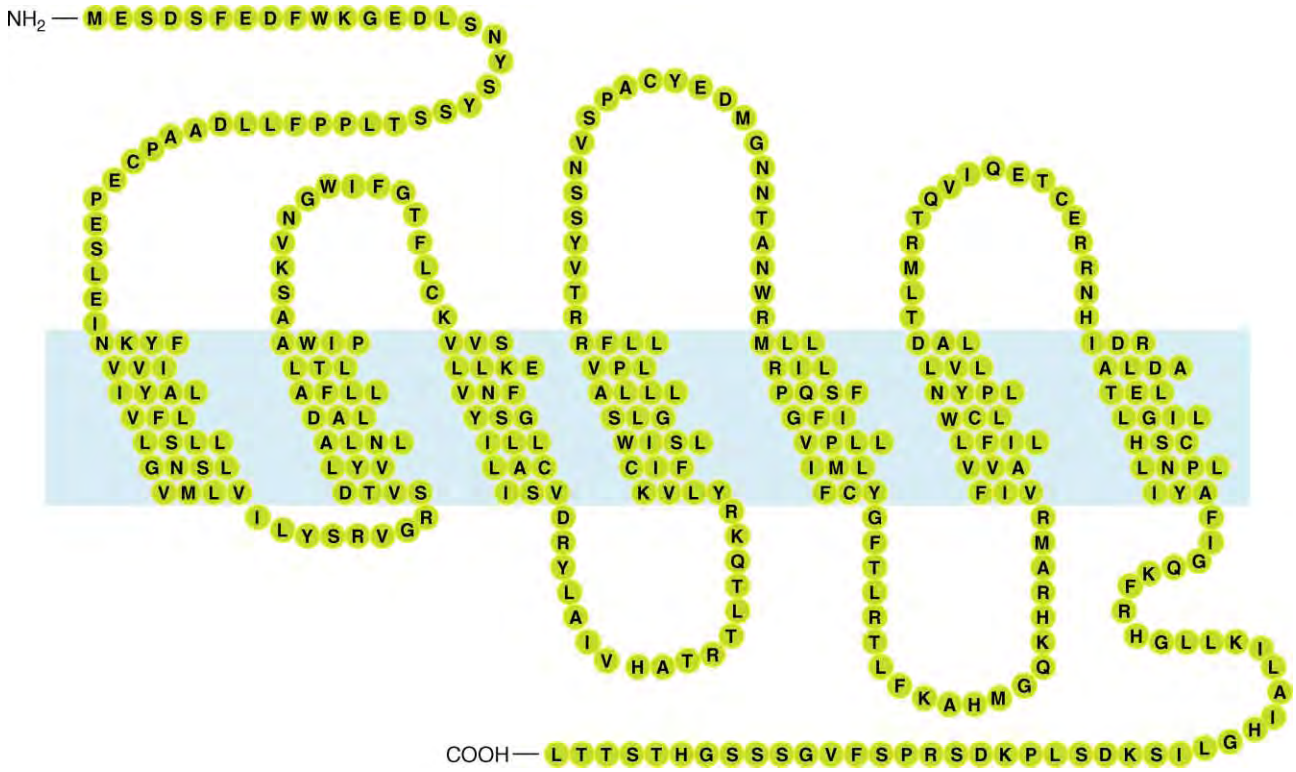



**FIGURE 1** Proposed conformation of the chemokine receptor CXCR2. This receptor is a seven-transmembrane G protein-coupled receptor. The free amino terminus provides ligand binding specificity. The third extracellular loop participates in ligand binding, whereas the third intracellular loop is thought to be the site of G protein coupling. The serine residues in the carboxyl terminus are phosphorylation sites.

## Desensitization

The ligand-induced activation of chemokine receptors is transient. The functional response of chemokine receptors is rapidly reduced following repeated stimulation by chemokines. This process is called desensitization. Agonist-dependent (homologous) desensitization occurs when a receptor binds chemokine and is phosphorylated by a G protein receptor-coupled receptor kinase (GRK). The phosphorylated receptor recruits $\beta$-arrestin, an adaptor protein that uncouples the receptor with G proteins, thus retaining the receptor in a desensitized state, resulting in attenuation in the cell excitability (Figure 3). This mechanism plays a major role in determining the duration of leukocyte trafficking, migration, or sequestration in certain situations (see later discussion). Chemokine receptors can also be phosphorylated by a kinase, such as PKC, activated by a different signaling cascade. This is called heterologous desensitization. For example, the chemokine receptor CXCR1 or CXCR2 can be phosphorylated and desensitized through the activation of formyl peptide receptors by the tripeptide formyl-methionyl-leucyl-phenylalanine (fMLP). The importance of this phenomenon in inflammation and leukocyte trafficking is not completely clear, but the capacity for heterologous desensitization appears to be different among chemokine receptors, suggesting a hierarchy of chemokines.

## Internalization

After agonist occupancy, chemokine receptors are rapidly translocated from the cell membrane to the cytoplasm. This process is called receptor internalization. Chemokine receptors generally undergo internalization through clathrin-coated pits, a complex process that involves clathrin and adaptor proteins such as $\beta$-arrestins and adaptin-2. After pinching off from the cell membrane through a dynamin-dependent process, the clathrin-coated vesicles containing the internalized receptors fuse with early endosomal compartments. When there is a low pH value in the early endosomes, the receptors become dephosphorylated by a specific serine/threonine protein phosphatase such as protein phosphatase 2A. The dephosphorylated receptors are either transported to recycling endosomes and subsequently reexpressed on the cell surface after removal of the extracellular ligand or delivered to late endosomes and lysosomes for degradation in the continued presence of a high concentration of ligands. The intracellular trafficking processes are regulated by a number of molecules including the Ras-like GTPases (Rabs) (Figure 3). Such trafficking may be important for

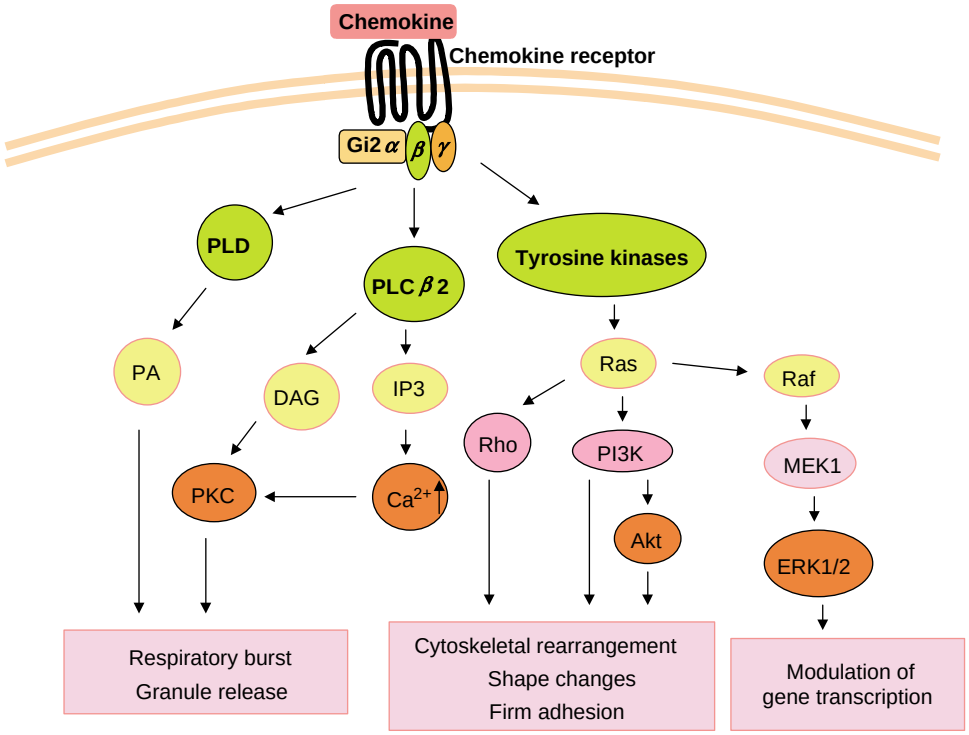



**FIGURE 2** The major intracellular signaling events induced by a chemokine binding to its receptor and activating responses in a neutrophil. Stimulation of a chemokine receptor by its ligand results in a conformational change of the receptor and activation of the coupled G proteins. The G$\alpha$ (proteins coupled with chemokine receptors are generally the inhibitory G$\alpha$ proteins (G$\alpha$i). The $\beta$- and $\gamma$-subunits of the G protein activate phospholipase C (PLC$\beta$2), which cleave PIP3 to produce diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 initiates mobilization of the intracellular free $Ca^{2+}$ and DAG activates PKC, which is required for the respiratory burst and granule release. Stimulation of chemokine receptors also results in activation of tyrosine kinases, which trigger several signaling pathways, including mitogen-activated protein kinase (MAPK) pathway, phosphotidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway, as well as Rho signaling pathway. These pathways are required for cell movement and gene transcription. In addition, stimulation of chemokine receptors results in activation of phospholipase D (PLD), which produces phosphatidic acid (PA), which plays a role in respiratory burst and granule release. ERK1/2, extracellular signal-regulated kinases 1 and 2; MEK, mitogen-activated protein kinase kinase; Ras and Rho, small G proteins.

both transmission and termination of the receptor signals and may play an important role in mediating cell chemotaxis (see later discussion).

## Multiple Roles

### Chemotaxis

Neutrophils, lymphocytes, and other immune cells migrate to the sites of injury and infection. This process, which is called chemotaxis, occurs through the dynamic response of chemokine receptors expressed on these cells to the gradient concentrations of chemokines produced in the inflammatory sites. Chemokine receptor signaling leading to the establishment of cell polarity, cytoskeletal rearrangement, and interaction with the extracellular matrix plays an essential role in this complex process. In addition, the desensitization and internalization of chemokine receptors play a regulatory role. For example, human immature dendritic cells derived from monocytes express the inflammatory chemokine receptors CXCR1, CCR1, CCR2, CCR5, and CCR7, which allow these cells to follow chemotactic gradients to inflammatory sites. Once there, dendritic cells process antigen and become exposed to the maturation-stimulating cytokines. Maturing dendritic cells express large amounts of chemokines such as CCL3, CCL4, CCL5, CCL8, and CCL10. One consequence of this increased chemokine expression is the down-regulation of chemokine receptors on maturing dendritic cells, particularly CCR1 and CCR5, by receptor desensitization and internalization. The second consequence of this up-regulation of chemokines is that it strengthens the original chemotactic gradient, further boosting recruitment of immature dendritic cells, monocytes, and lymphocytes.

### HIV Entry

Human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS). Type 1 HIV (HIV-1) uses chemokine receptor as a coreceptor together with CD4 to enter the CD4-positive (CD4+) target cells. The main cells targeted by HIV-1 are

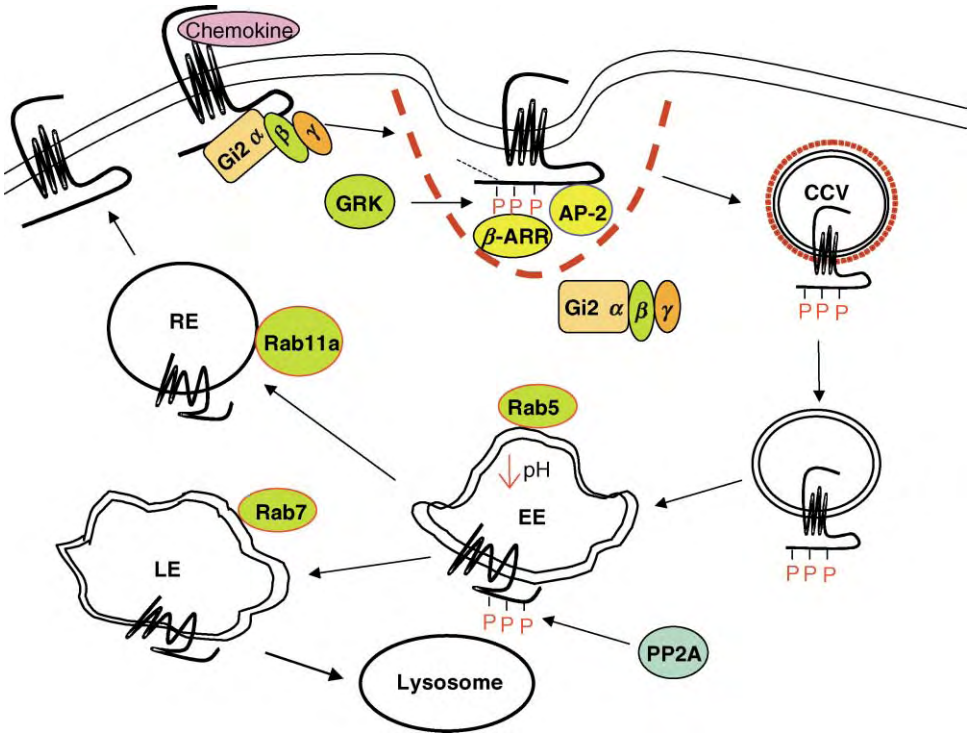



**FIGURE 3** Homologous desensitization and intracellular trafficking of chemokine receptors. In response to chemokine stimulation, the receptor undergoes homologous desensitization, in which phosphorylation by a G protein-coupled receptor kinase leads to uncoupling of G proteins, binding of β-arrestins, and subsequent internalization of the receptor through clathrin-coated pits. After pinching off from the membrane, the clathrin-coated vesicles fuse to early endosomes, where the chemokine receptors are dephosphorylated by protein phosphatase 2A, and the receptors are transported to late endosomes and lysosomes for degradation. However, after removal of the extracellular ligands, the receptors are transported from early endosomes to recycling endosomes so as to re-express on the cell surface. β-ARR, β-arrestin; AP2, adaptin 2; CCV, clathrin-coated vesicle; EE, early endosome; GRK, G protein-coupled receptor kinase; LE, late endosome; P, phosphate; PP2A, protein phosphatase 2A; Rab5, Rab11a, Rab7, small GTPases that regulate receptor trafficking; RE, recycling endosome.

T lymphocytes (T cells), macrophages, and probably dendritic cells. The chemokine receptors CXCR4 and CCR5 are the major coreceptors for T-cell line-tropic and macrophage-tropic HIV-1 strains, respectively, although many other chemokine and orphan receptors have also been identified as potential coreceptors for HIV-1. The fusion of the viral envelope and the lymphocyte is as follows. The envelope glycoprotein on the surface of virus particles comprising a trimer of three gp120 and three transmembrane gp41 molecules binds to CD4 and triggers a structural change, which exposes a binding site for a coreceptor. Further structural rearrangements are initiated when the coreceptor is bound. These changes occur predominantly in gp41 and are sufficient to trigger fusion of viral and cellular membranes and entry of the virion core into the cytoplasm.

## Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing microvasculature. It is a biological process that is critical to both physiological and pathological processes such as wound healing and tumor growth. Angiogenesis is regulated by an opposing balance of angiogenic and angiostatic factors. The CXC chemokines are a family of cytokines unique in their ability to behave in a disparate manner in the regulation of angiogenesis. The N terminus of the majority of the CXC chemokines contains three amino acid residues (Glu-Leu-Arg; the ELR motif). This motif, in general, determines whether these chemokines promote angiogenesis. Members that contain the ELR motif (ELR+), such as CXCL1 and CXCL8, are potent promoters of angiogenesis. In contrast, members that are inducible by interferons and lack the ELR motif (ELR−), such as CXCL10, are potent inhibitors of angiogenesis. ELR-CXC chemokines bind to CXCR2 and a few to CXCR1, whereas non-ELR-CXC chemokines bind to CXCR3, CXCR4, CXCR5, and CXCR6. The mechanisms for the differential regulation of angiogenesis by chemokines and chemokine receptors are under investigation.

## Tumor Growth and Metastasis

Many cancer cells such as melanomas express a number of chemokines, including CXCL8, CXCL1-3, CCL5,

and CCL2, which have been implicated in tumor growth and progression. Recent studies have demonstrated organ-specific patterns of melanoma metastasis that correlate with their expression of specific chemokine receptors, including CXCR4, CCR7, and CCR10. The chemokine receptors CXCR4 and CCR7 are also found on breast cancer cells and their ligands are highly expressed at sites associated with breast cancer metastases. Other models in which CXCR4 has been suggested to play a role in metastasis are ovarian, prostate, and lung cancers. It is thus postulated that chemokine receptors and their ligand pairs may play a role in the migration of tumor cells from their primary site via the circulation to the preferential sites of metastases.

## Central Nervous System

In addition to their well-established role in the immune system, chemokine receptors also play a role in the development and inflammation of the central nervous system (CNS). Certain chemokine receptors such as CXCR2, CXCR4, CCR1, and CCR5 are constitutively expressed in both developing and adult brains, and the role played by these proteins in the normal brain is the object of intense study. Chemokines are involved in brain development and in the maintenance of normal brain homeostasis; these proteins play a role in the migration, differentiation, and proliferation of glial and neuronal cells. For example, the chemokine receptor CXCR4 is essential for life during development, and this receptor has a fundamental role in neuron migration during cerebellar formation. In the CNS, chemokines play an essential role in neuroinflammation as mediators of leukocyte infiltration and glial cell activation. Their overexpression has been implicated in several neurological disorders, such as multiple sclerosis, trauma, stroke, Alzheimer's disease, tumor progression, and AIDS-associated dementia.

## See Also the Following Articles

G Protein-Coupled Receptor Kinases and Arrestins • Phosphatidylinositol Bisphosphate and Trisphosphate • Phosphoinositide 3-Kinase • Phospholipase C • Protein Kinase C Family • Ras Family

## Glossary

**angiogenesis** The growth of new vasculature from pre-existing blood vessels.

**chemotaxis** Directed migration of leukocytes toward to an inflammatory site.

**coreceptor** Any of certain chemokine receptors that are used together with CD4 by human immunodeficient virus to enter the target cells.

**desensitization** Attenuation of the functional response of chemokine receptors to their ligands following repeated ligand stimulation.

**internalization** Translocation of chemokine receptors from the cell membrane to the cytoplasmic compartments.

**metastasis** The migration of cancer cells to sites distant from the primary tumor.

## Further Reading

Bajetto, A., Bonavia, R., Barbero, S., and Schettini, G. (2002). Characterization of chemokines and their receptors in the central nervous system: Physiopathological implications. *J. Neurochem.* **82**, 1311–1329.

Belperio, J. A., Keane, M. P., Arenberg, D. A., Addison, C. L., Ehlert, J. E., Burdick, M. D., and Strieter, R. M. (2000). CXC chemokines in angiogenesis. *J. Leukoc. Biol.* **68**, 1–8.

Dhawan, P., and Richmond, A. (2002). Role of CXCL1 in tumorigenesis of melanoma. *J. Leukoc. Biol.* **72**, 9–18.

Murphy, P. M., Baggiolini, M., Charo, I. F., Hébert, C. A., Horuk, R., Matsushima, K., Miller, L. H., Oppenheim, J. J., and Powe, C. A. (2000). International Union of Pharmacology, XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev.* **52**, 145–176.

Olson, T. S., and Ley, K. (2002). Chemokines and chemokine receptors in leukocyte trafficking. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **283**, R7–R28.

Richmond, A. (2002). NF-kappa B, chemokine gene transcription and tumour growth. *Nat. Rev. Immunol.* **2**, 664–674.

Wang, J-M., Deng, X., Gong, W., and Su, S. (1998). Chemokines and their role in tumor growth and metastasis. *J. Immunol. Methods* **220**, 1–17.

## Biography

Ann Richmond is a Professor in the Department of Cancer Biology at Vanderbilt University School of Medicine and a Senior Career Scientist in the Department of Veterans Affairs at Nashville Campus. Her research interests include chemokine receptor signal transduction, desensitization, and trafficking; transcriptional regulation of chemokines; and biological roles of chemokines in tumorigenesis, angiogenesis, and wound healing. She received her Ph.D. from Emory University and her postdoctoral training in the Division of Endocrinology at Emory University. During that time, she and her colleagues characterized one of the first chemokines, which is now known as CXCL1.

Guo-Huang Fan is a Research Assistant Professor in the Department of Cancer Biology at Vanderbilt University and an Assistant Professor in the Department of Pharmacology at Meharry Medical College. His research interests are signaling and trafficking of chemokine receptors. He received his Ph.D. from Shanghai Second Medical University and his postdoctoral training at the Chinese Academy of Sciences, University of Wuerzburg, and Vanderbilt University.

# Chemolithotrophy

**Alan B. Hooper**
*University of Minnesota, St. Paul, Minnesota, USA*

**The chemolithotrophic bacteria are defined by their ability to utilize inorganic or one-carbon molecules as a growth-supporting reductant. Oxidation of the growth-supporting reductant initiates a chain of redox electron-transfer reactions ending with reduction of a terminal electron acceptor such as oxygen and resulting in the generation of ATP. Alternatively, the reducing equivalents from the growth-supporting substrate are used in biosynthetic reductive reactions leading to cellular components.**

## Bioenergetics

### Oxidation of the Growth-Supporting Substrate Drives Synthesis of Adenosine Triphosphate (ATP)

In all organisms a source of energy is required to drive biosynthesis, mechanical work, and pumping of molecules across a membrane against a gradient. Within cells this "energy" is commonly manifest as ATP since the hydrolysis of its terminal phosphoric anhydride bond can drive an otherwise thermodynamically unfavorable reaction when the two reactions are functionally coupled by an enzyme. The rephosphorylation of the resulting adenosine diphosphate (ADP) is most often catalyzed by a remarkable ATP synthase, an enzyme that is able to use the potential energy of a steady-state transmembrane gradient of protons. As the protons are relayed through the protein and across the membrane, the process is coupled to the synthesis of ATP. In turn, maintenance of the proton gradient requires the continuous turnover of a "low potential" half reaction (the oxidation of the growth-supporting reducing substrate). The continuation of the latter oxidative reaction is dependent on a "high potential" half reaction by which a growth-supporting *oxidant* substrate (a terminal electron acceptor) is reduced by electrons originating from the growth supporting reductant. A series of enzymes and electron transfer proteins are arranged in the membrane so that the process results in the net transfer of protons to the external side against a proton gradient.

### The Variety of Terminal Electron Acceptors Used by Bacteria

Many bacteria are able to use one or more of the half-reactions shown in Table I as an electron sink supporting the continuity of the energy-generating reactions (i.e., the substrate molecules of Table I are terminal electron acceptors). The name of the enzyme is given for half-reactions: (1a–1g) are half-reactions; (1h–1k) are identified as processes. Because of its high redox potential, half-reaction 1a is preferred by bacteria. The other terminal electron acceptors are used when oxygen is absent (i.e., in anoxic or anaerobic rather than oxic or aerobic environments). Note that the reduction of protons in half-reaction 1f requires a low-potential reductant. Half-reactions 1f and 1g are employed in anaerobic fermentation by organotrophs. Line 1h is a multistep process carried out by the methane-generating bacteria. In line 1i, each step involves two electrons. The sulfur-reducing bacteria vary in the steps they are able to use. The reactions in this table are steps in the cycling of carbon, hydrogen, nitrogen, sulfur, and metals in nature.

### Reductant for Biosynthesis

Biosynthetic reactions often require an electron or hydrogen donor molecule with a low enough potential to drive reductive reactions. This is often the reduced form of nicotinamide adenine dinucleotide (NADH). Hence, a second role of the growth-supporting reductant is the reduction of $NAD^+$; $NAD^+ + 2e^- + H^+ \rightarrow$ NADH.

## Lithotrophy

### Categories of Growth Based on the Nature of the Growth-Supporting Reductant

Organisms for which the growth-supporting reductant is a reduced carbon molecule such as glucose are called *chemoorganotrophs*. The sum of many reactions, including dehydrogenations, in the pathway of aerobic

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Reductive Reactions Employed by Bacteria[a]**

| Half reaction | Name of enzyme or process |
|---|---|
| Aerobic conditions | |
| a. $O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$ | cytochrome oxidase |
| Anaerobic conditions | |
| b. $NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$ | nitrate reductase |
| c. $NO_2^- + 2e^- + 2H^+ \rightarrow NO + H_2O$ | nitrite reductase |
| d. $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$ | nitric oxide reductase |
| e. $N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$ | nitrous oxide reductase |
| f. $2H^+ + 2e^- \rightarrow H_2$ | hydrogenase |
| g. $CH_3CHO^- + 4e^- + 4H^+ \rightarrow CH_3CH_2OH + H_2O$ | aldehyde dehydrogenase |
| h. $CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O$ | methanogenesis |
| i. $SO_4^{2-} \rightarrow SO_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow S_2O_3^{2-} \rightarrow S^0 \rightarrow S^{2-}$ | reduction of sulfate |
| j. $Fe^{3+} + e^- \rightarrow Fe^{2+}$ | |
| k. $Mn^{4+} + 2e^- \rightarrow Mn^{2+}$ | |

[a] The substrates of these reactions are terminal electron acceptors.

glucose oxidation is $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$.

Organisms defined as *phototrophs* are able to photochemically activate an internal substituent of a membrane protein thus converting it to a lower potential reductant. Reoxidation of the latter drives the creation of the proton gradient. Electrons are then recycled back to the center of photochemical reduction. Phototrophs require an external source of reductant (e.g., $H_2O$) only for reduction of NAD for use in biosynthetic reactions.

Bacteria that are specialized to use inorganic or one-carbon compounds as their growth-supporting reductant are called *chemolithotrophs*. Bacteria that are only able to grow in this manner are *obligate* chemolithotrophs; *facultative* chemolithotrophs have the activities necessary for chemoorganotrophic or chemolithotrophic growth.

The reducing half-reactions of the major chemolithotrophic growth-supporting substrates are listed in Tables II–V. In the majority of chemolithotrophic bacteria the oxidation of the growth-supporting reductant is coupled to reduction of oxygen (Table I, line a). The overall reactions supporting ATP synthesis for aerobic bacteria which oxidize ammonia, methane, sulfur, hydrogen, or ferric ion, respectively, are as follows:

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+$$

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$$

$$S^0 + 3O_2 \rightarrow SO_4^{2-} + 2H^+ + 2H_2O$$

$$2H_2 + O_2 \rightarrow 2H_2O$$

$$4Fe^{2+} + 4H^+ + O_2 \rightarrow 4Fe^{3+} + 2H_2O$$

Bacteria that use a substrate half-reaction with a sufficiently low redox potential can grow anaerobically using terminal electron acceptors other than oxygen:

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$$

$$4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O$$

$$5S_2O_3^{2-} + 8NO_3^- + H_2O \rightarrow 10SO_4^{2-} + 4N_2 + 2H^+$$

## Examples of Half-Reactions of the Growth-Supporting Reductant in the Chemolithotrophic Bacteria

### Nitrifying Bacteria

The half-reactions of the nitrifying bacteria represent the oxidative arm of the nitrogen cycle. Two enzymes are involved in the ammonia-oxidizers and one enzyme is involved in the nitrite oxidizers, respectively (see Table II). A recently discovered anaerobic chemolithotroph generates energy in a series of ANAMMOX

**TABLE II**

**Oxidation of the Growth-Supporting Reductant in the Nitrifying Bacteria**

| Half reaction | Name of enzyme |
|---|---|
| Ammonia oxidizers | |
| overall growth-supporting half-reaction | |
| a. $NH_3 + O_2 \rightarrow HNO_2 + H_2O + 2H^+ + 2e^-$ | |
| enzyme steps | |
| b. $NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH$ | ammonia monooxygenase |
| c. $NH_2OH + H_2O \rightarrow HNO_2 + 4e^- + 4H^+$ | hydroxylamine oxidoreductase |
| Nitrite oxidizers | |
| d. $HNO_2 + H_2O \rightarrow HNO_3 + 2H^+ + 2e^-$ | nitrite oxidoreductase |
| Anaerobic conversion of ammonia to dinitrogen (ANAMMOX bacterium) | |
| overall reaction supporting energy conservation | |
| e. $HNO_2 + NH_3 \rightarrow N_2 + 2H_2O$ | |
| enzyme steps | |
| f. $NO_2^- + 4e^- + 4H^+ \rightarrow NH_2OH + H_2O$ | (hypothetical) |
| g. $NH_2OH + NH_3 \rightarrow N_2H_2 + H_2O$ | (hypothetical) |
| h. $N_2H_4 \rightarrow N_2 + 4e^- + 4H$ | hydroxylamine oxidoreductase |

**TABLE III**

**Oxidation of the Growth-Supporting Reductant in the Hydrogen-, Methane-, and Carbon Monoxide-Oxidizing Bacteria**

| Half reaction | Name of enzyme or process |
|---|---|
| Hydrogen-oxidizing bacteria | |
| a. $H_2 \rightarrow 2H^+ + 2e^-$ | hydrogenase |
| Methanotrophic bacteria | |
| overall growth-supporting half-reaction: | |
| b. $CH_4 + 2O_2 \rightarrow CO_2 + 4H^+ + 4e^- + 2H_2O$ | |
| enzyme steps | |
| c. $CH_4 + O_2 + 2H^+ + 2e^- \rightarrow CH_3OH + H_2O$ | methane monooxygenase |
| d. $CH_3OH \rightarrow CH_2O + 2H^+ + 2e^-$ | methanol dehydrogenase |
| e. $CH_2O + H_2O \rightarrow H_2CO_2 + 2H^+ + 2e^-$ | formate dehydrogenase |
| f. $H_2CO_2 + H_2O \rightarrow H_2CO_3 + 2H^+ + 2e^-$ | formate dehydrogenase |
| g. $H_2CO_3 \rightarrow CO_2 + H_2O$ | carbonic anhydrase |
| Carboxydobacteria | |
| h. $CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$ | CO dehydrogenase |

reactions involving ammonia and nitrite with hydrazine as an intermediate and dinitrogen as the end product.

## Hydrogen-, Methane-, and Carbon Monoxide-Oxidizing Bacteria

Hydrogenase (Table I, line f) of hydrogen-oxidizing bacteria oxidizes hydrogen to protons. The aerobic oxidation of methane by the methanotrophic bacteria (Table III, line b) involves an electron consuming monooxygenase (line c) and successive methanol-, formaldehyde-, and formate- dehydrogenases (lines d–f). The conversion of carbonic acid to $CO_2$ can be spontaneous or enzyme-catalyzed (line g). In the carboxydobacteria, carbon monoxide reductase catalyzes the net extraction of two electrons and two protons to produce carbon dioxide from carbon monoxide and water.

**TABLE IV**

**Oxidation of the Growth-Supporting Reductant in the Sulfur Oxidizing Bacteria**

| Half reaction |
|---|
| Overall growth-supporting half-reactions |
| a. $H_2S + 4H_2O \rightarrow SO_4^{2-} + 10H^+ + 8e^-$ |
| b. $S_2O_3^{2-} + 5H_2O \rightarrow 2SO_4^{2-} + 10H^+ + 8e^-$ |
| c. $S^0 + 4H_2O \rightarrow SO_4^{2-} + 8H^+ + 6e^-$ |
| d. $SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$ |
| Enzymatic steps observed within sulfur oxidizing pathways |
| e. $H_2S \rightarrow S^0 + 2H^+ + 2e^-$ |
| f. $2S^0 + 3H_2O \rightarrow S_2O_3^{2-} + 6H^+ + 4e^-$ |
| g. $2S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 2e^-$ |
| h. $S_4O_6^{2-} + 6H_2O \rightarrow 4SO_3^{2-} + 12H^+ + 12e^-$ |
| i. $SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$ |

**TABLE V**

**Oxidation of the Growth-Supporting Reductant in the Metal Oxidizing Bacteria**

| Half reaction |
|---|
| a. $Fe^{II} \rightarrow Fe^{III} + e^-$ |
| b. $Mn^{II} \rightarrow Mn^{III} + e^-$ |
| c. $Cu^{I} \rightarrow Cu^{II} + e^-$ |
| d. $Sn^{II} \rightarrow Sn^{III} + e^-$ |
| e. $Fe^0 \rightarrow Fe^{II} + 2e^-$ |

## Sulfur Oxidizing Bacteria

Depending on the specific bacterium, oxidation of a variety of reduced sulfur compounds are used to support growth. Some examples of the half-reactions are shown in Table IV, lines a–d. The enzymatic steps used within the respective pathways are listed in Table IV, lines e–i. Some bacteria utilize the adenosine phosphosulfate (APS) pathway for synthesis of ATP coupled to the oxidation of sulfite.

## Metal-Oxidizing Bacteria

The half reactions shown in Table V, lines a–d are carried out by *Thiobacillus ferrooxidans*. A metalloenzyme catalyzes the oxidation of ferrous iron to ferric ion which hydrolyzes to form an orange precipitate: $Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+$. Metallic $Fe^0$ can be oxidized by methanogenic bacteria coupled to the reduction of $CO_2$ to methane (Table I, line h).

# Basic Mechanisms of Energy-Transduction and ATP Synthesis in Chemolithotrophs

## Generation of the Electrochemical Gradient and ATP Synthesis

Some generalizations regarding location of the enzymes catalyzing the oxidation of substrate, generation of a proton gradient and synthesis of ATP are illustrated in

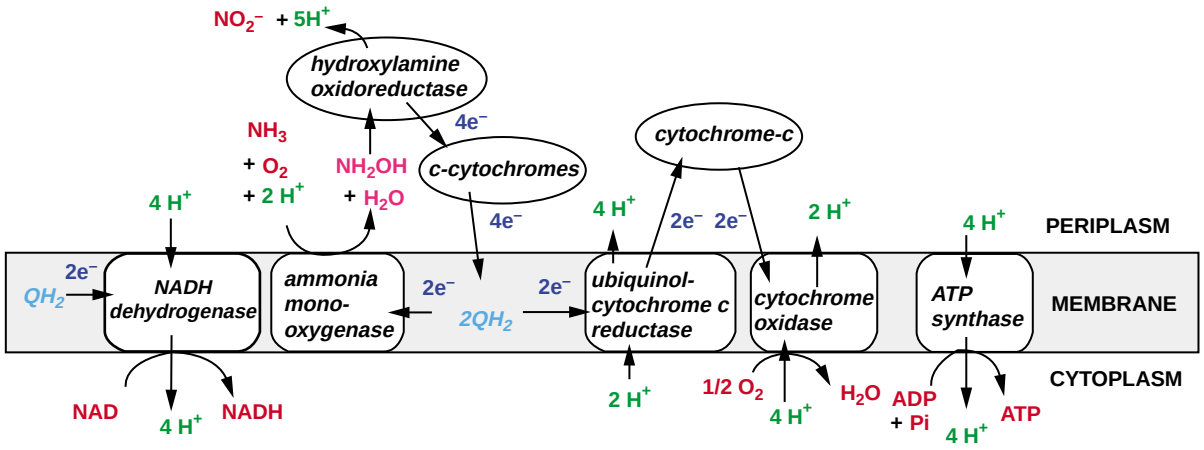



**FIGURE 1** Catalysis of the oxidation of ammonia to nitrite coupled to generation of ATP and NADH in the chemolithotrophic bacterium, *Nitrosomonas europaea*. The four enzymes in the center of the figure create a proton gradient that is employed by the enzymes on the left and right to make NADH and ATP, respectively. Electrons from hydroxylamine oxidoreductase are relayed by heme-containing electron-transferring proteins, "cytochromes" in which Fe cycles between $Fe^{2+}$ and $Fe^{3+}$, to reduce a quinone to a quinol ($QH_2$). The quinol is reoxidized either by ammonia monooxygenase or by an electron-transfer chain including a proton-pumping ubiquinol–cytochrome c reductase, a cytochrome- c and a proton-pumping cytochrome oxidase. The proton-pumping NADH-dehydrogenase functions in reverse to form NADH as shown in the figure. ATP synthase catalyzes the proton-gradient-driven phosphorylation of ADP. The periplasm is the region outside the cell membrane; the cytoplasm is inside the cell membrane. The membrane itself is impermeable to protons. Substrates and final products are shown in red (the intermediate hydroxylamine is pink). Protons are shown in green. Electrons are shown in blue and quinone is in light blue. Enzymes and cytochromes are italicized and circled.

the oxidation of ammonia to nitrite by the nitrifying bacterium *Nitrosomonas*, an obligate chemolithoautotroph (Figure 1). Although ammonia is the substrate required for growth, it is the oxidation of hydroxylamine that generates low-potential electrons for energy conversion and reductive biosynthetic reactions. Four electrons are transferred from hydroxylamine oxidoreductase (Table II, line c) to an electron-transfer chain made up of two heme-containing proteins (cytochromes) to two quinone molecules in the membrane. Two electrons from the reoxidation of one quinol are utilized by ammonia monooxygenase to regenerate a molecule of hydroxylamine, i.e., to "re-prime the pump." The two electrons (per hydroxylamine oxidized) from the second quinol may reduce dioxygen in a pathway that involves the two membrane enzymes ubiquinol cytochrome c reductase and cytochrome c oxidase, each of which couple the reaction to the transport of protons against a gradient to the outside (the "periplasm" of the bacterial cell). In this way a steady-state electrochemical potential (outside positive) is maintained across the membrane and "drives" ATP synthesis.

## Substrate-Level Phosphorylation of ADP

In one chemolithotroph, a sulfite oxidizing bacterium, the net phosphorylation of ADP occurs by a substrate-level process that is independent of the membrane electrochemical gradient. The key reaction involves the simultaneous dehydrogenation of sulfite and reaction with adenosine monophosphate (AMP) to form the anhydride of phosphoric and sulfuric groups in the product, adenosine phosphosulfate (APS).

$$SO_3^{2-} + AMP \rightarrow APS + 2e^-$$

Phosphate can then displace sulfate to form ADP and the subsequent dismutation of two molecules of ADP provides an ATP.

$$APS + P_i \rightarrow ADP + SO_4^{2-}$$

$$2ADP \rightarrow ATP + AMP$$

## Release and Uptake of Protons in Chemolithotrophic Reactions

In the reducing half-reactions of the major chemolithotrophic growth-supporting reductants (Tables II–V) the energetically fruitful reactions are, in effect, dehydrogenases where the products are free protons and electrons transferred to a metal redox center. The protons originate from the substrate (in the oxidation of ethanol, formaldehyde, $H_2$, $N_2H_2$, $H_2S$), $H_2O$ (in the oxidation of CO, nitrite, $S^0$, $SO_3^{2-}$) or both (in the oxidation of $NH_2OH$). In all cases where location of the enzyme has been determined, the proton-yielding reactions are found in the periplasm and thus contribute to maintenance of the positive charge on the outer side of the membrane. Correspondingly, almost all terminal electron acceptor reactions (Table I) consume protons and are located on the internal side of

the membrane thus contributing to the maintenance of the negative charge on that side. This is illustrated in the location of hydroxylamine dehydrogenation and oxygen reduction in *Nitrosomonas* (Figure 1).

### Production of NADH by Chemolithotrophic Bacteria

With many chemolithotrophs, the potential of the half reaction that drives metabolism (in the case of *Nitrosomonas*, the dehydrogenation of hydroxylamine) is not low enough to reduce $NAD^+$ directly. The reduction of $NAD^+$ is catalyzed by an enzyme which, in chemoorganotrophs, dehydrogenates NADH, reduces quinone, and pumps protons to the outside of the cell. However, in the case of *Nitrosomonas* (Figure 1) and other chemolithotrophs, the reaction runs in the reverse direction; thermodynamically, the steady-state gradient of protons drives the reduction of $NAD^+$ by quinol. This process is not needed in bacteria such as the hydrogen oxidizers in which the growth-supporting reductive half reaction ($H_2 \rightarrow 2H^+ + 2e^-$) has a low enough potential to reduce $NAD^+$ directly.

## Carbon Source for Chemolithotrophic Bacteria

The carbon source for most chemolithotrophic bacteria is $CO_2$. This mode of growth is called *autotrophic*, i.e., the organisms are chemolithoautotrophs. Most chemolithotrophs are *obligate* autotrophs, meaning that they can only grow having synthesized all or most of their reduced carbon compounds from $CO_2$. This contrasts with heterotrophic organisms, which can fulfill all their need for carbon compounds with previously fixed carbons such as sugars. *Facultative* autotrophs are able to alter their content of biosynthetic enzymes to catalyze either the autotrophic or heterotrophic mode of life. The most common pathway of carbon dioxide fixation in the chemolithoautotrophs is the Calvin Cycle starting with the reaction of ribulose bis phosphate carboxylase:

$$\text{ribulose 1,5 bis phosphate} + CO_2 \rightarrow 2,3\text{-phosphoglycerate}$$

Considering the reduction of 3-phosphoglycerate and the regeneration of ribulose 1,5 bis phosphate in the Calvin cycle, approximately 12 molecules of NADH and 18 of ATP are required per hexose synthesized from $CO_2$. Remarkably, the obligate chemolithoautotrophs contain the enzymes for biosynthesis of all cellular constituents. Furthermore, they have genes for enzymes for the uptake, metabolism, and incorporation of carbon from extremely few carbon compounds other than $CO_2$. This is illustrated nicely by analysis of the genes present and absent in the genome of *Nitrosomonas*.

## Growth Yields of Chemolithoautotrophs

Given the required expenditure of energy (in effect, low potential electrons from the substrate) to drive the synthesis of ATP and the reduction of NAD, which are then used in biosynthetic reactions, the yield of biomass per mole of substrate oxidized is usually much lower in the chemolithoautotrophs than with heterotrophic organotrophs. Profiting from their unique catalytic capabilities, chemolithotrophs grow slowly and live frugally with a highly selective diet of a thin soup of inorganic compounds or small carbon molecules.

## Ecology

The chemolithotrophs represent the oxidative segment of the biological cycles of inorganic compounds such as hydrogen, nitrogen, sulfur, and metal ions. The vast majority of these steps occur aerobically. Most of the reduced substrates for chemolithotrophs are generated by the terminal electron accepting reactions of anaerobic organisms (Table I). For example, hydrogen from reduction of protons by organotrophic bacteria in cattle rumen (Table I, line f) is oxidized by chemolithotrophic hydrogen-oxidizing bacteria using the conversion of carbon dioxide to methane as the terminal electron acceptor half-reaction (line h). Methane released from ruminants finds its way to an oxic (aerobic) environment, where it is oxidized by the methanotrophic bacteria (Table III, line b). The chemolithotrophs and the anaerobes are sometimes found at oxic/anoxic (aerobic/anaerobic) interfaces, where the respective oxidative and reductive reactions provide a cycle to the advantage of both types of organism.

There are many examples of economically significant effects of chemolithotrophic activity. The production of sulfuric acid by the sulfur-oxidizing bacteria facilitates corrosion of metals and the acidification of mine drainage is attributed to the iron oxidizing bacteria.

Other major natural sources of the reduced compounds include volcanic activity and atmospheric activity such as lightning. Anthropogenic sources of reduced compounds include agricultural waste and residential waste water, combustion of fossil fuels, industrial processes, and mining operations. Significantly, the annual production of ammonia from dinitrogen by industry now exceeds the flux from all other biological and nonbiological sources.

## See Also the Following Article

Respiratory Processes in Anoxygenic and Oxygenic Phototrophs

## Glossary

**autotrophy** A mode of bacterial growth in which carbon dioxide is the source of all or most of the carbon for biosynthesis. These bacteria are *autotrophic*. Autotrophy contrasts with heterotrophy, a mode of growth where organic carbon compounds can provide all carbon for biosynthesis.

**chemolithotrophy** A mode of bacterial growth in which the reductive substrate for energy generation and for biosynthesis is an inorganic or a single carbon compound. These bacteria are *chemolithotrophic*. Chemolithotrophy contrasts with organotrophy or phototrophy in which the energy generation occurs by the oxidation of organic molecule or absorption of light, respectively.

**growth-supporting reductant** A compound whose oxidation can be coupled biologically to the conservation of energy in forms of general use to cellular activities: as an electrochemical gradient, as a phosphate anhydride bond in ATP, or as the low-potential reductant, reduced nicotinamide adenine dinucleotide.

**oxic environment** An environment containing dioxygen ($O_2$). The word *aerobic* is also used. Oxic contrasts with anoxic (or anaerobic), which applies to environments in which oxygen is absent.

**terminal electron acceptor** A compound whose reduction can be coupled to the stoichiometric oxidation of the growth-supporting reductant and conservation of energy in cells.

## Further Reading

Chain, P., Lamerdin, J., Larimer, F., Regala, W., Lao, V., Land, M., Hauser, L., Hooper, A., Klotz, M., Norton, J., Sayavedra-Soto, L., Arciero, D., Hommes, N., Whittaker, M., and Arp, D (2003). Complete genome sequence of the ammonia oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *J. Bacteriol*. **185**, 2759–2773.

Hooper, A. B., and DiSpirito, A. A. (1985). In bacteria which grow on simple reductants, generation of a proton gradient involves extracytoplasmic oxidation of substrate. *Microbiol. Rev.* **49**, 140–157.

Hooper, A. B., Arciero, D. M., Bergmann, D., and Hendrich, M. P (2003). The oxidation of ammonia as an energy source in bacteria in respiration. In *Archaea and Bacteria, Volume 2: Diversity of Procaryotic Respiratory Systems* (D. Zannoni, ed.), in press. Kluwer Scientific, The Netherlands.

Lengeler, J. W., Drews, G., and Schlegel, H. G. (1999). *Biology of the Prokaryotes*. Blackwell Science, Oxford, U.K.

White, D. (2000). *The Physiology and Biochemistry of Prokaryotes*. Oxford University Press, New York.

## Biography

Alan Bacon Hooper is a Professor in the Department of Biochemistry, Molecular Biology and Biophysics at the University of Minnesota. His research interest is in the biochemistry of the ammonia oxidizing chemolithotrophic bacteria. He earned his B.A. from Oberlin College and his Ph.D. from Johns Hopkins University. He joined the University of Minnesota faculty in 1963. He currently teaches biochemistry and a course on Biology, Ethics, and Public Policy.

# Chemotactic Peptide/Complement Receptors

**Eric R. Prossnitz and Larry A. Sklar**
*University of New Mexico, Albuquerque, New Mexico, USA*

**A critical aspect in the normal function of many cell types is their ability to migrate in response to soluble ligands or chemoattractants. Directional migration, where the cell seeks out the highest concentration of chemoattractant, is referred to as chemotaxis. This is of paramount importance in the immune system where a large collection of G protein-coupled receptors (GPCRs) exists to coordinate the complex interactions of immune cells. These receptors can be divided into two families, the chemotactic or chemoattractant GPCRs that respond to a wide variety of endogenous as well as exogenous ligands and the chemokine GPCRs that respond to the structurally conserved family of endogenous peptide chemokines. The chemotactic GPCRs respond to ligands as varied as oligopeptides, such as bacterially derived N-formylated peptides; proteins, such as complement components; and lipids, such as leukotrienes and platelet-activating factor.**

## Leukocytes and the Immune System

The immune system is responsible for numerous activities in the body including defense against pathogens (bacterial, viral, and parasitic), removal of dead and cancerous cells as well as the rejection of foreign cells. Leukocytes, or white blood cells, which represent the principal cells of the immune system, are produced as progenitor cells in the bone marrow and mature in various sites throughout the body, including the spleen, thymus, and lymph nodes. Leukocytes can be divided into five broad categories: neutrophils (highly mobile phagocytic cells), basophils (allergy mediators through histamine release), eosinophils (parasitic killing and allergy), lymphocytes (B cells, which produce antibodies and T cells, which effect cell-mediated immunity), and monocytes/macrophages (tissue-specific phagocytic cells). These cells differentially participate in two types of immune responses: innate immunity, an inherent and rapid response of neutrophils, macrophages, and natural killer lymphocytes to foreign materials; and acquired immunity, a selective "trained" response involving lymphocytes requiring prior exposure to the agent. An additional noncellular defensive mechanism, the complement system consisting of at least 20–30 distinct serum proteins, destroys foreign cells through membrane lysis of antibody-targeted cells. Two of the smaller bioactive fragments released by proteolysis from the C3 and C5 components, C3a and C5a, are known as anaphylatoxins and play a major role in inflammation. Immune cells for the most part circulate in the vascular and lymphatic systems, although tissue resident cells such as macrophages are essential to proper immune function. In almost any immune response, however, leukocytes must migrate to the site of damage/infection from the blood. This is a complex multistep process involving inflammatory mediators (such as histamine) that induce vasodilation and increased vascular permeability, activation of adhesion molecules on capillary lining endothelial cells to slow leukocytes and release of chemoattractants to activate leukocytes resulting in firm adhesion to endothelial cells. The concentration gradients of chemoattractants induce the leukocyte to transverse the endothelium and migrate through the tissue to the peak concentration of chemoattractant at the site of inflammation or infection. Having arrived at this site, neutrophils, for example, phagocytose bacteria, generate superoxide radicals and release granules containing degradative proteases. Most of these responses are initiated and regulated by a family of cell surface receptors that bind inflammatory mediators.

## Receptors

The largest family of proteins in the human genome is the GPCR superfamily consisting of at least 600 members. GPCRs exhibit a highly conserved structure, consisting of seven transmembrane segments with the amino terminus on the cell exterior and the carboxy terminus in the cytoplasm. The chemoattractant family of receptors consists of proteins with ~350 amino acids. The transmembrane regions consist primarily of $\alpha$-helices arranged in a bundle. Despite this conserved

 © 2004, Elsevier Inc. All Rights Reserved. 

structure, there is substantial sequence diversity between members of this receptor family. Binding of small ligands, such as chemotactic peptides, occurs deep within the receptor in the plane of the membrane. Ligand binding and receptor activation result in a conformational change that activates heterotrimeric G proteins, which further stimulate a wide variety of effector proteins. Following activation, GPCRs undergo phosphorylation, resulting in desensitization and internalization, which in turn can lead to receptor degradation or recycling back to the cell surface.

### Chemotactic Peptide Receptors

Though identified as a neutrophil receptor for formyl peptides in the 1970s, it was not until 1990 that the FPR gene was isolated, representing the first chemoattractant or chemokine receptor to be cloned. Two human homologues were subsequently identified and designated FPRL1 and FPRL2. Of these, only the former responds, though poorly, to formyl peptides. Although the FPR is expressed predominantly in neutrophils, it also appears to be expressed in monocytes, dendritic cells, hepatocytes, endothelial cells, and the nervous system. FPRL1 and FPRL2 are expressed in some of the same cell types as well as unique sites. Although the human FPR gene family consists of only three members, the number varies considerably in other species, with six members in the murine gene family for example. Thus, distinct evolutionary pressures seem to dictate the repertoire of formyl peptide receptors in a given organism.

The role of the FPR in antimicrobial defense has recently been directly demonstrated in knockout mice lacking mFPR1, the homologue of the human FPR. Although such mice appear normal under unstressed conditions, they demonstrate a lack of neutrophil responses to fMLF challenge as well as an increased susceptibility to infection with *Listeria monocytogenes*. Furthermore, in humans, two variant alleles of the FPR representing single point mutations that disrupt normal function are associated with localized juvenile periodontitis, a result of periodontal bacterial infection. It is perhaps surprising that such defects do not lead to more serious conditions.

### Complement Receptors

#### C5a Receptor

At the same time that formylated peptides were being characterized as chemoattractants, proinflammatory peptides derived from the complement system were similarly being studied. Two receptors have been identified that respond to the cleavage products of the complement components C3 and C5. The C5a receptor is similar in size to the FPR and closely related phylogenetically. Interestingly, there exists little interspecies homology in the extracellular regions of the C5a receptor, despite apparent contact sites with C5a and interspecies activation (C5a from one species activating the receptor from another species). Antibodies against peptides representing the amino terminus of the receptor and amino-terminal truncations of the receptor block C5a binding. As the antibodies do not block activation of the receptor by synthetic C5a carboxy terminal peptides, the amino terminus of the receptor may represent a secondary noneffector binding site and not the primary effector site. Mutagenesis studies suggest a critical role for a number of aspartic acid residues in the amino terminus. Recent evidence is consistent with C5a receptor dimerization, a phenomenon now demonstrated for many GPCRs with unclear functional consequences. Studies of genetically engineered mice lacking the C5a receptor demonstrated that this receptor plays a critical role in clearing pulmonary pseudomonas infections, with mice succumbing to pneumonia despite a marked neutrophil influx.

#### C3a Receptor

The C3a receptor is unusual in that it possesses a second extracellular loop of some 175 amino acids compared to about 30 amino acids for most GPCRs including the C5a and FPRs. Deletion analysis of this loop suggests that it plays a role in C3a binding and receptor activation, although as much as two-thirds of the loop can be deleted without affecting activity. It is thought that the second extracellular loop may replace the function of the amino terminus of the C5a receptor in providing a secondary noneffector binding site. Recent studies with C3a receptor knockout mice suggest a role for the receptor in allergic airway diseases such as asthma.

## Ligands

### Chemotactic Peptide Receptors

The first leukocyte chemotactic factors to be defined structurally were the N-formyl peptides. These consist of short oligopeptides consisting of 3–6 hydrophobic amino acids with the amino terminal residue being formylated. Such peptides are not synthesized by eukaryotic cells *per se*. There are only two sources of N-formylated peptides in nature: one endogenous to eukaryotic cells, mitochondria, and the other exogenous, bacteria. Each of these sources initiates protein synthesis with an N-formyl methionine tRNA. This suggests an ideal targeting mechanism for leukocytes to sense bacterial infection or cell damage through the release of peptides containing N-formyl-methionyl peptides.

The first chemoattractant peptide was isolated from bacterial lysates and was identified in 1976 as N-formyl-methionyl-leucyl-phenylalanine (fMLP or fMLF). This agonist is capable of eliciting the full complement of leukocyte functions from chemotaxis to degranulation to superoxide production at concentrations ranging from pM to nM. The FPR is the primary receptor responsible for cellular stimulation in these concentration ranges, whereas FPRL1 responds to fMLF at μM concentrations. Both the FPR and FPRL1 have recently been shown to be activated by a diverse collection of endogenous as well as exogenous peptides. These include non-formylated peptides from exogenous sources such as Helicobacter pylori and HIV-1 envelope proteins as well as endogenous host-derived proteins such as serum amyloid A and peptides from annexin I, amyloid precursor protein and prion protein among others. Lipoxin A4, a lipid metabolite, has also been reported to activate FPRL1. The biological relevance of these agonists remains to be determined. Finally, a number of non-formylated high-affinity peptide agonists for the FPR and FPRL1 have been isolated from peptide libraries. A number of antagonists have also been identified including Boc-FLFLF, cyclosporine H (a fungal metabolite), and deoxycholic acid (a bile acid).

### Complement Receptors

#### C5a

The anaphylatoxin C5a, the most potent plasma-derived chemotactic factor, is a 74 amino acid peptide/protein released from the $\alpha$-chain of C5 by either the classical or alternative pathway C5 convertases. Structurally, the protein consists of four antiparallel $\alpha$-helices held together by disulfide bonds and an 11-amino acid carboxy terminal tail. Unlike the small formyl peptides, C5a appears to interact with its receptor at two sites. The $\alpha$-helical bundle interacts with the receptor's amino-terminal domain whereas the tail appears to insert itself into the transmembrane bundle of the receptor, thereby activating the receptor. Synthetic peptides representing the carboxy-terminal 10-amino acids are full agonists, though with potencies 1000–10 000 lower than C5a itself. The sequence Gln–Leu–Gly–Arg is a highly conserved effector sequence in C5a from numerous species. Substitution of residues within this region can greatly augment potency. Removal of the final arginine residue in C5a results in greatly decreased potency, ranging from 1000-fold decrease for spasmogenic activity to tenfold for chemotactic activity.

#### C3a

Less is known regarding the interactions between C3a and its receptor. C3a is a 77-amino acid protein released from the $\alpha$-chain of C3 by either the classical or alternative pathway C3 convertases. C3a and C5a, despite having common genetic ancestry, have markedly different sequences. Only 13-amino acid positions have been totally conserved between C3a, C5a, and the related C4a molecules from numerous species analyzed. Six of these residues represent immutable cysteine residues that direct folding of the protein. Again, the carboxy-terminal sequence seems to define molecular action as the terminal sequence Leu–Gly–Leu–Ala–Arg is conserved in all known C3a molecules. C3a appears to interact both with portions of the large second extracellular loop of the receptor as well as charged residues at the transmembrane boundaries of this loop. For both the C5a and C3a receptors, insertion of the carboxy terminus of the anaphylatoxin into a binding pocket formed by the transmembrane helices of the receptor appears essential to receptor activation.

## G Proteins

Following ligand binding and the commensurate activation of the receptor, GPCRs interact with G proteins initiating a vast array of intracellular signaling (Figure 1). Of the four major G protein subfamilies ($G_s$, $G_i$, $G_q$, and $G_{12/13}$), chemoattractant/complement receptors activate primarily the $G_i$ family of G proteins. This is demonstrated by the inhibition of virtually all chemoattractant receptor-mediated cell activation by pertussis toxin, a bacterial toxin that inactivates $G_i$ (and closely related $G_o$) $\alpha$-subunits of G proteins. G proteins consist of three subunits: the guanine nucleotide-binding $\alpha$-subunit, and the nondissociable membrane-associated $\beta\gamma$-subunit dimer. Receptor activation catalyzes the exchange of GDP for GTP on the $\alpha$-subunit followed by activation of the $\alpha$- and $\beta\gamma$-subunits. Hydrolysis of the bound GTP by the intrinsic activity of the $G_\alpha$ protein, accelerated by a family of regulators of G protein signaling (RGS) proteins, inactivates the protein and allows reassembly of the inactive $\alpha\beta\gamma$ heterotrimer.

## Effector Proteins

Chemoattractant-mediated G protein activation leads to a plethora of downstream signaling events. Although $G_i$ proteins are generally considered to inhibit adenylyl cyclase activity, in the case of most chemoattractant and chemokine receptors, $G_i$ activation leads to $G_{\beta\gamma}$-mediated activation of phospholipase C$\beta$2, resulting in the production of inositol trisphosphate (IP3) and diacyl glycerol (DAG). IP3 generation results in the release of calcium from intracellular endoplasmic reticulum stores, often followed by the influx of

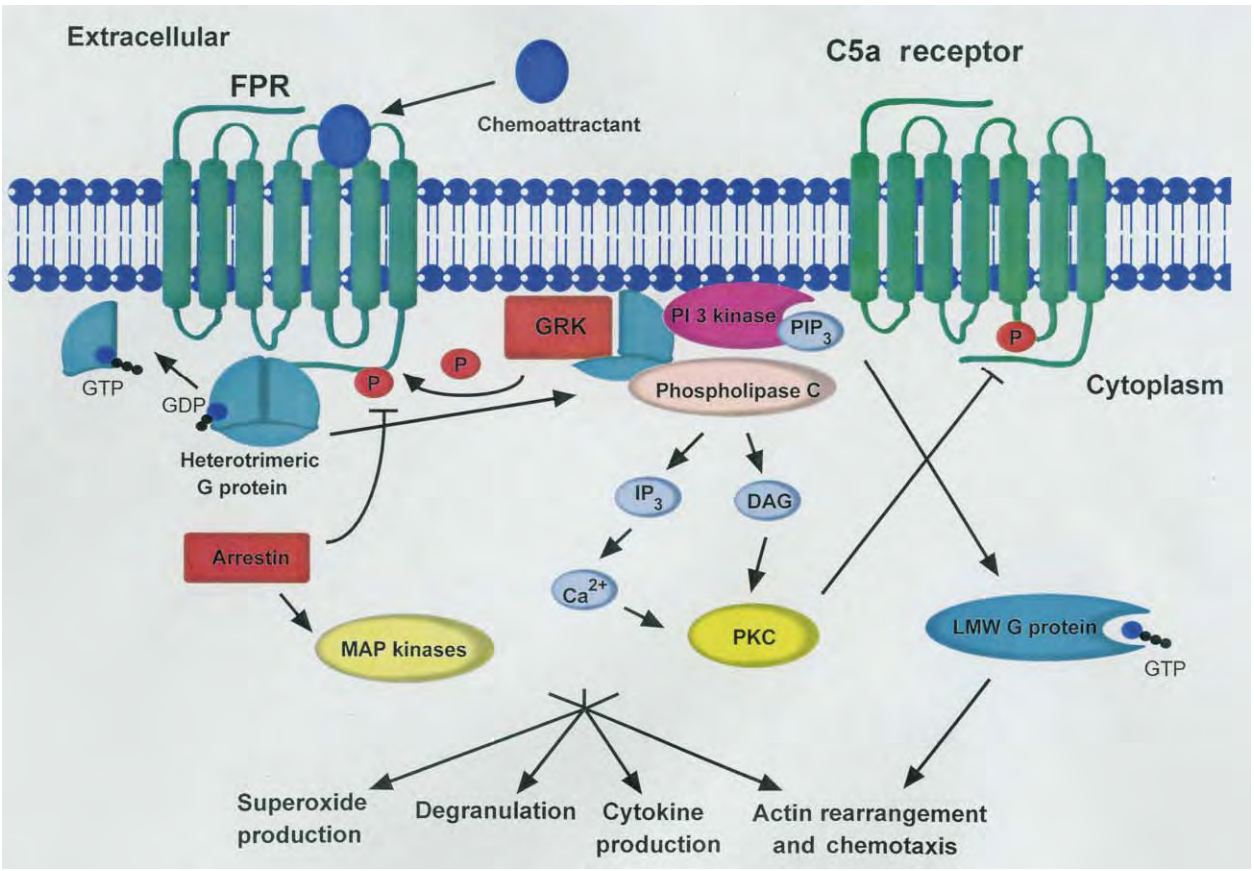



**FIGURE 1** Signaling and regulation of chemoattractant/complement receptors in leukocytes. Binding of a chemoattractant (agonist) results in a conformational change in the FPR, which activates G proteins. Dissociation of GDP from the $\alpha$-subunit is followed by GTP binding and dissociation of the $\alpha$-subunit from the $\beta\gamma$-subunits, both of which can stimulate downstream effectors. The $\beta\gamma$-subunits provide a docking site for G protein-coupled receptor kinases (GRKs), which phosphorylate (P) the ligand-bound, activated receptor. $\beta\gamma$-subunits also activate phospholipases, which generate diacyl glycerol (DAG) and inositol trisphosphate ($IP_3$) and phosphatidylinositol-3 kinase ($PI_3$ kinase), which generates phosphatidyl inositol trisphosphate ($PIP_3$). $PIP_3$ is an activator of numerous pathways including those culminating in low molecular weight (LMW) monomeric G proteins of the Ras superfamily that regulate actin assembly and ultimately chemotaxis. $IP_3$ production results in the release of $Ca^{2+}$ from intracellular stores, and together with DAG activates protein kinase C (PKC). PKC is capable of phosphorylating many proteins including complement receptors, such as the C5a receptor, resulting in their heterologous desenstization. Desensitization of the FPR occurs through the binding of arrestin to GRK-phosphorylated receptors. In addition to preventing further signaling through heterotrimeric G proteins, arrestins serve as scaffolds for multiple MAP kinases, resulting in their localized activation. Together, these and many additional pathways produce cellular responses including superoxide generation, degranulation, chemotactic cell movement and transcriptional activation resulting in cytokine production.

calcium from the extracellular environment through ion channels. The calcium and DAG in turn activate protein kinase C (PKC). Receptor-mediated phospholipase $A_2$ activation results in the generation of arachidonic acid and subsequently prostaglandins and leukotrienes. Phosphatidylinositol 3-kinase (PI3K) also plays a critical role in leukocyte activation and chemotaxis in particular. MAP kinase family members ERK, p38, and Jnk have all been shown to be essential to cellular activation events. The low-molecular-weight monomeric G proteins Rac and Rap, as well as their associated regulatory proteins and downstream effectors, are important mediators of actin assembly/chemotaxis and superoxide production.

## Receptor Regulation

The dysregulated activation of leukocytes is a serious contributing factor to numerous chronic inflammatory diseases such as arthritis and reperfusion injury in ischaemic tissues following myocardial thrombosis and stroke. Activated chemoattractant/complement receptors are rapidly phosphorylated following ligand binding and receptor activation. This can be mediated either by G protein-coupled receptor kinases (GRKs) that are specific for the active state of GPCRs or by second messenger kinases such as protein kinase A and protein kinase C that phosphorylate active and inactive receptors. The latter provides a mechanism

(termed heterologous desensitization) by which GPCRs such as the FPR, which has no PKC phosphorylation sites, can desensitize the C5a receptor, which is phosphorylated by PKC, leading to decreased G protein coupling (see Figure 1). Since any inflammatory process is likely to lead to the generation of multiple simultaneous chemoattractant signals, these regulatory mechanisms create a hierarchy of chemotactic responsiveness to different ligands. GRK phosphorylation of chemoattractant receptors, which occurs within the carboxy terminus of the receptor at sites distinct from PKC sites, leads to binding of arrestins and receptor internalization. Arrestin binding further uncouples receptors from G proteins by physically preventing access of G proteins to the receptor (termed homologous desensitization). For many GPCRs, arrestin binding also mediates interactions with clathrin and adapter proteins such as AP-2 initiating receptor translocation to clathrin-coated pits and subsequent internalization. Contrary to this paradigm, the FPR has recently been shown to internalize in an arrestin-independent manner. However, arrestin is required for recycling of the FPR. Arrestins have also been shown to play an important role in the scaffolding of numerous intracellular signaling cascades including Src and MAP kinases. This mechanism is believed to provide both spatial and temporal regulation of the downstream effectors.

## Drug Discovery and Receptor Technology

GPCRs represent ~50% of the targets of drugs on the market today. In particular, the family of chemokine/chemoattractant receptors is a highly sought after target as the receptors are involved in immune disorders such as chronic inflammation and asthma, cancer, and many more disease states. The formyl peptide receptor, in particular, has served as an important model for the real-time analysis of ligand-receptor interactions based on the detection of fluorescent peptide ligands by solution fluorescence, flow cytometry, and microscopy. These ligands and receptors have provided the basis for developing subsecond kinetic resolution of molecular assemblies and disassemblies by flow cytometry as well as small volume, high throughput flow cytometric platforms for screening. These approaches are now being extended to other molecular classes.

### See Also the Following Articles

Chemokine Receptors • Chemotactic Peptide/Complement Receptors • G Protein Signaling Regulators • G Protein-Coupled Receptor Kinases and Arrestins • $G_i$ Family of Heterotrimeric G Proteins • Protein Kinase C Family

### Glossary

**chemokine** A family of structurally related 70–90 aminoacid-long glycoproteins that exhibit potent chemotactic and stimulatory properties on leukocytes.

**complement system** A series of ~30 components that include proteolytic pro-enzymes, non-enzymatic components that form functional complexes, regulators, and receptors. The proteolytic pro-enzymes become sequentially activated. C5a and C3a are terminal non-enzymatic cleavage products.

**G protein** A protein that binds and is regulated by guanine nucleotides. Heterotrimeric members of this family couple to GPCRs. Low-molecular-weight monomeric members of the family are activated downstream of heterotrimeric G proteins.

**G protein-coupled receptor (GPCR)** Transmembrane receptor containing seven membrane-spanning helices that activates cells through coupling to heterotrimeric G proteins.

### Further Reading

Cicchetti, G., Allen, P. G., and Glogauer, M. (2002). Chemotactic signaling pathways in neutrophils: From receptor to actin assembly. *Crit. Rev. Oral Biol. Med.* **13**, 220–228.

Ember, J. A., and Hugli, T. E. (1997). Complement factors and their receptors. *Immunopharmacology* **38**, 3–15.

Haribabu, B., Richardson, R. M., Verghese, M. W., Barr, A. J., Zhelev, D. V., and Snyderman, R. (2000). Function and regulation of chemoattractant receptors. *Immunol. Res.* **22**, 271–279.

Le, Y., Murphy, P. M., and Wang, J. M. (2002). Formyl-peptide receptors revisited. *Trends Immunol.* **23**, 541–548.

Prossnitz, E. R., and Ye, R. D. (1997). The N-formyl peptide receptor: A model for the study of chemoattractant receptor structure and function. *Pharmacol. Ther.* **74**, 73–102.

Sklar, L. A., Edwards, B. S., Graves, S. W., Nolan, J. P., and Prossnitz, E. R. (2002). Flow cytometric analysis of ligand-receptor interactions and molecular assemblies. *Ann. Rev. Biophys. Biomol. Struct.* **31**, 97–119.

### Biography

Dr. Eric R. Prossnitz is Associate Professor of Cell Biology and Physiology at the University of New Mexico Health Sciences Center. His principal research interest is in the broad field of G protein-coupled receptor activation and regulation, utilizing the N-formyl peptide receptor of leukocytes as a model system. He holds a Ph.D. from the University of California at Berkeley and received his postdoctoral training at the Scripps Research Institute.

Dr. Larry A. Sklar is Regents' Professor of Pathology at the University of New Mexico Health Sciences Center. His principal interests are in signal transduction and cell adhesion molecular assemblies. He holds a Ph.D. from Stanford University and received postdoctoral training at the Baylor College of Medicine.

# Chlorophylls and Carotenoids

**Hugo Scheer**
*Universität München, Munich, Germany*

**The green (Greek "chloros") of leaves (Greek "phyllos") is a mixture of two water-insoluble pigment classes: chlorophylls, mainly the blue chlorophyll (Chl) *a*, and carotenoids, mainly *β*-carotene. Both pigment classes cooperate in photosynthesis to safely capture light provided by the Sun, as the primary energy source for life on Earth. Chlorophylls are responsible for light harvesting and its transduction to an electrochemical potential across the photosynthetic membrane which ultimately serves to reduce atmospheric carbon dioxide to carbohydrates. Thus, the energy of the fleeting photons is stored in increasingly longer-lived products: first Chl excited states are generated with lifetimes in the order of several nanoseconds ($10^{-9}$ s), then a membrane potential which is stable for seconds, then high-energy products such as ATP and NADH that live for minutes or even hours, and eventually storage products like starch.**

**The carotenoids can act, too, as light-harvesting pigments, in regions where Chl has only little absorption. Their major and indispensable function is, however, to safely drain excess excitation energy from the chlorophylls and convert it into heat. Photons are by biological standards a high-energy source which is potentially highly toxic (sunburn). The combination of chlorophylls with carotenoids allows photosynthetic organisms a careful balance between tapping and competing for this source, and being killed by an overdose. Photosynthesis based on these two pigments has therefore conquered nearly all habitats on earth where light is available. It has produced the atmospheric oxygen we breathe, and fixes $\sim 10^{11}$ ton of carbon annually. The greening and degreening of the vegetation is probably the most obvious life-process on Earth, visible clearly from outer space. The widespread occurrence of photosynthesis is paralleled by considerable variations of the photosynthetic apparatus, including the pigments. It is this variety, but also the basic similarities among the different chlorophylls and carotenoids, respectively, which will be dealt with in this article.**

## Chlorophylls

### Structures

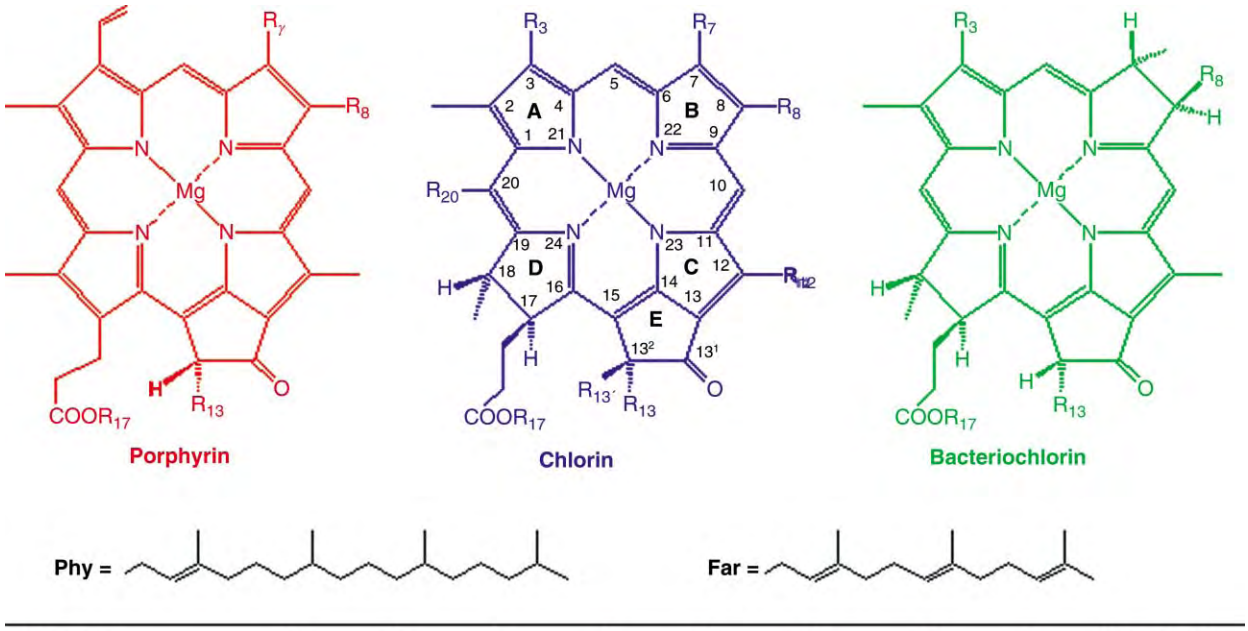
The basic structure of chlorophylls is the porphyrin macrocycle. It is comprised of four pyrrole rings containing each four carbons and one nitrogen, which are joined via one-carbon bridges to an aromatic macrocycle (Figure 1). The four nitrogens face inward, creating a hole which is ideal for binding metal ions. In the chlorophylls, this central metal is almost always magnesium (Mg). While many of the peripheral substituents vary among the different structures, all chlorophylls contain an additional five-membered ring E and, with the exception of some Chl *c*, the C-17 propionic acid side chain is esterified by a long-chain alcohol, usually the $C_{20}$ terpenoid alcohol, phytol. The chlorophylls can further be classified by the degree of unsaturation of the macrocylus, into the fully unsaturated true porphyrins, the chlorins with an unsaturated ring D and the bacteriochlorins in which ring B is unsaturated, too.

### Spectroscopy

The spectral properties of chlorophylls are described by the four-orbital model: chlorophylls have four major absorptions termed $Q_Y$, $Q_X$, $B_Y$, and $B_X$, in the near ultraviolet (NUV), visible (Vis), and near infrared (NIR) spectral regions. The type of spectrum is mainly determined by the degree of unsaturation of the tetrapyrrole macrocycle (Figure 2): in the fully unsaturated Chl *c* containing a porphyrin macrocyclic system, the B-bands around 400 nm overlap, the $Q_Y$-band around 620 nm is weak, and $Q_X$-band is visible only with special techniques. In chlorin-type chlorophylls (Chl *a, b, d,* BChl *c, d, e*), the B-bands are reduced in intensity, the $Q_X$-band increased to near equal intensity and redshifted to $\sim$660 nm. In the bacteriochlorin-type chlorophylls (BChl *a, b, g*), the B-bands are blueshifted to $<$400 nm and well separated, the $Q_y$-band is even more increased in intensity and redshifted to $\sim$780 nm, and in these pigments the $Q_y$-band also has gained in intensity and becomes visible around 570 nm. By these characteristics, the chlorophyll type can be readily determined from the spectra, and often also other details like substitution pattern, ligation to the central metal (e.g., by the protein), or aggregation.

Chlorophylls dissolved in organic solvents have long-lived excited states ($10^{-8}$ s). They are therefore highly fluorescent, and there is significant intersystem

 © 2004, Elsevier Inc. All Rights Reserved. 




| Pigment | $R_3$ | $R_7$ | $R_8$ | $R_{12}$ | $R_{13'}$ | $R_{13}$ | $R_{17}$ | $R_{20}$ | Macrocycle |
|---|---|---|---|---|---|---|---|---|---|
| Chl *a* | $C_2H_3$ | $CH_3$ | $C_2H_5$ | $CH_3$ | H | $COOCH_3$ | Phy | H | Chlorin |
| Chl *b* | $C_2H_3$ | CHO | $C_2H_5$ | $CH_3$ | H | $COOCH_3$ | Phy | H | Chlorin |
| Chl *d* | CHO | $CH_3$ | $C_2H_5$ | $CH_3$ | H | $COOCH_3$ | Phy | H | Chlorin |
| Phe *a* | $C_2H_3$ | $CH_3$ | $C_2H_5$ | $CH_3$ | H | $COOCH_3$ | Phy | H | Chlorin |
| BChl *c* | $CHOH\text{-}CH_3$ | $CH_3$ | $C_2H_5$ | $CH_3/C_2H_5$ | H | H | Far + others | $CH_3$ | Chlorin |
| BChl *d* | $CHOH\text{-}CH_3$ | $CH_3$ | $C_2H_5$ | $CH_3/C_2H_5$ | H | H | Far + others | H | Chlorin |
| BChl *e* | $CHOH\text{-}CH_3$ | $CH_3$ | $C_2H_5$ | $CH_3/C_2H_5$ | H | H | Far + others | $CH_3$ | Chlorin |
| BChl *a* | $CO\text{-}CH_3$ | $CH_3$ | $C_2H_5$ | $CH_3$ | H | $COOCH_3$ | Phy + others | H | Bacteriochlorin |
| BChl *b* | $CO\text{-}CH_3$ | $CH_3$ | $=CH\text{-}CH_2$ | $CH_3$ | H | $COOCH_3$ | Phy + others | H | Bacteriochlorin |
| BChl *g* | $CO\text{-}CH_3$ | CHO | $=CH\text{-}CH_2$ | $CH_3$ | H | $COOCH_3$ | Phy + others | H | Bacteriochlorin |
| Chl(ide) $c_1$ | $C_2H_3$ | $CH_3$ | $C_2H_5$ | $CH_3$ | H | $COOCH_3$ | H[a,b1] | H | Porphyrin |
| Chl(ide) $c_2$ | $C_2H_3$ | $CH_3$ | $C_2H_3$ | $CH_3$ | H | $COOCH_3$ | H[a,b1] | H | Porphyrin |
| Chl(ide) $c_3$ | $C_2H_3$ | $COOCH_3$ | $C_2H_5$ | $CH_3$ | H | $COOCH_3$ | H[a,b1] | H | Porphyrin |
| [8-Vinyl]-PChlide *a* | $C_2H_3$ | $CH_3$ | $C_2H_3$ | $CH_3$ | H | $COOCH_3$ | H[a,b1] | H | Porphyrin |

a) Sometimes esterified, b) Acrylic side chain at c-$17^1$-$17^2$ double bond)

**FIGURE 1** Structures of common chlorophylls. In particular, reaction centers contain a series of specialized chlorophylls which are being dealt with in a recent survey by M. Kobayashi and co-workers in 1999.

crossing to the triplet state, giving rise to phosphorescence and, more significantly, under aerobic conditions to the generation of highly reactive oxygen species (ROS) like singlet oxygen. Chlorophyll solutions therefore bleach rapidly by attack of the pigment by these ROS, and chlorophylls injected into animals lead to severe "sunburn" and destruction of tissue. This effect is used in photodynamic therapy (PDT) of cancer.

In chlorophyll aggregates, there is generally a pronounced redshift of the $Q_Y$-band, and the excited state lifetimes (and thereby fluorescence and phosphorescence) are drastically reduced due to rapid conversion of the excitation energy into heat by internal conversion (IC). These effects are summarized under the term "concentration quenching" and probably due to the presence of "traps" in the large aggregates in which the excitation is highly delocalized over many pigment molecules.

Aggregation is also observed in photosynthetic pigment–protein complexes, where it is considered a major organizing force. In addition, there is a general redshift of all bands brought about by the protein environment. There is, however, a very distinct difference to aggregates: while excitation energy is mainly transformed to heat in the latter, this loss is avoided in the pigment protein complexes. Isolated light harvesting complexes (LHC), which contain the vast majority ($\geq 99\%$) of chlorophylls, therefore show, under moderate light intensities, high fluorescence. The avoidance of concentration quenching in these aggregates is in fact one of the major, mechanistically still unresolved tricks of efficient photosynthesis. In the reaction centers

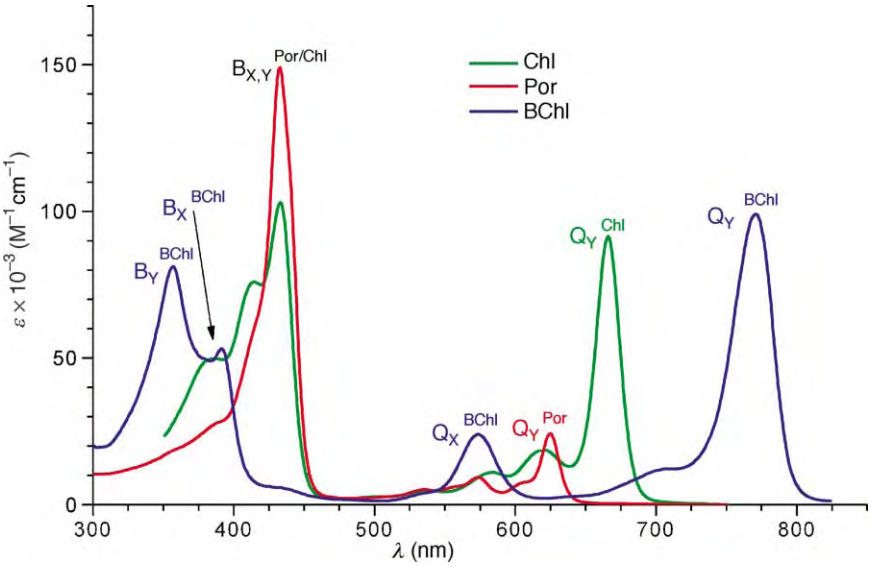



**FIGURE 2** Type spectra of the three chlorophyll classes: porphyrins (Por), chlorins (Chl), and bacteriochlorins (BChl).

(RCs), the lifetime is short, but here the excitation energy is used to create, with quantum efficiencies near 100%, a charge separation across the photosynthetic membrane which is otherwise an insulator, thus creating a long-lived membrane potential. This energy transduction process is at the heart of photosynthesis, and the high-resolution crystal structures of RC together with extensive spectroscopy at high time-resolution have provided a general picture on how this process is working. In the photosynthetic apparatus, LHC containing >99% of the chlorophylls are coupled to RC, such that now the excitation energy of the former is efficiently transferred to the latter. By this means, the absorption is increased by orders of magnitude, and different numbers and types of LHC can be combined to adapt to the prevailing light conditions. Since the amount of protein is much less in LHC than in RC, the biosynthetic expenses for such a coupled system are at the same time strongly reduced.

## Occurrence and Functions

The majority of chlorophylls, both in terms of structure diversity and quantity, serve as light-harvesting pigments. Taken together, they absorb light across most of the spectral range from 350 to 1020 nm, with a gap only in the region from 500 to 600 nm where carotenoids and biliproteins add to light harvesting. No organism contains all types of chlorophylls, however, so different organisms occupy different spectral niches, and the pigmentation is an important taxonomic criterion. Due to the more demanding energetics, oxygenic organisms (plants, algae, cyanobacteria) can only use light <730 nm, while the anoxygenic photosynthetic bacteria have specialized in the NIR with extremes like the purple bacterium, *Blastochloris viridis*; reaching 1020 nm. RCs contain chlorophylls, too, including besides the bulk pigments some specialized derivatives. They form a chain of pigments across the membrane, over which in a stepwise fashion electrons are transferred from the primary donor, a pair of chlorophylls located on the periplasmic side of the membrane, to the acceptors on the cytoplasmic side. It should be mentioned that some chlorophyll-like pigments perform other functions in nature: certain deep-sea fish use chlorophylls as visual pigments, and in the marine worm, *Bonella viridis*, a chlorophyll-derivative acts as a sex determinant.

## Chemical Properties

Chlorophylls are large, aromatic molecules with a central hole fit to complex many metals. They are basically planar, but not rigid, and deviations from planarity are observed in most structures of chlorophyll proteins. While the macrocyclic system is quite stable, the functional groups and the central Mg render the chlorophylls quite labile. Under acidic conditions, the central Mg is lost rapidly, and the long-chain alcohol residue hydrolyzed. The central Mg is also readily replaced by other, more stable metals like Zn, Ni, Ag, Cu. Under alkaline conditions, the isocyclic ring is modified extensively. The macrocycle can be opened at the methine bridges, both (photo)chemically and enzymatically.

After binding to the tetrapyrrole, the central Mg still has free valences, which are important for aggregation, and in particular for interactions with the natural protein environment. By interactions with suitable amino acids (e.g., histidin, methionin, glutamate) they can be positioned properly for efficient energy or electron transfer. In the LHC of green bacteria, the chlorosomes, the coordinating properties of the central Mg and the ligation of special peripheral substituents

combine to form large aggregates of bacteriochlorophylls *c*, *d*, and *e* nearly devoid of protein.

### Metabolism

The formation of chlorophylls can be separated in stages. The first is the synthesis of 5-aminolevulinic acid (ALA), the basic building block of all tetrapyrroles. In most organisms it is formed from glutamic acid, involving an intermediate (Glu-tRNA$^{glu}$) which is otherwise only involved in protein synthesis. Some photosynthetic bacteria (purple bacteria) use, like man, the alternative $C_{4+1}$-pathway in which succinyl coenzyme A is condensed with glycin. The second stage is ubiquitous in all organisms and for all natural tetrapyrroles. The two ALA first react to yield a pyrrol (porphobilinogen), a five membered ring containing one nitrogen atom. Four such pyrroles then condense to a linear tetrapyrrole, and then cyclize in a remarkable reaction in which one of the pyrroles is flipped. The resulting cyclic tetrapyrrole is transformed by a series of decarboxylation and oxidation reactions to protoporphyrin. It is noteworthy that the latter is the first colored product in the synthesis, and highly phototoxic like most porphyrins. The organisms therefore avoid, over large stretches of biosynthesis, the formation of such potentially harmful precursors. They also regulate precisely the amount of ALA in order to avoid any overproduction of protoporphyrin, and any deregulation of this process, as any alteration of the biosynthesis can result in severe damage and often death. The last stage of the pathway is the insertion of Mg, which is thermodynamically unfavorable and energy requiring, and the formation of the isocyclic ring from the propionic acid side chain at C-13. While the synthesis of Chl *c* is practically complete at this stage, all other chlorophylls require further modifications, viz., the reduction of ring D (chlorins) and B (bacteriochlorins), modifications at the periphery, and the condensation of the C-17 propionic acid with the long-chain alcohol derived from the isoprenoid pathway.

Chlorophyll degradation has been studied in some detail for Chl *a* and *b*, it involves the (light independent) ring-opening to the much less phototoxic open-chain tetrapyrroles (bilins). Very little is known on the degradation of the other pigments.

### Applications

Unmodified chlorophylls are too labile for most practical use, but some derivatives are used as dyes for cosmetics and food (Cu-chlorophyllin), and in photodynamic tumor therapy (chlorins, bacteriochlorins). The "chlorophyll" used, e.g., in certain health care is a complex mixture of degradation products. The best source for Chl *a* is the cyanobacterium *Spirulina platensis*, which is available commercially. Chl *a*/*b* mixtures can be obtained from all green plants. All other chlorophylls are less readily accessible, thereby limiting their applications.

## Carotenoids

### Structures

Carotenoids much more widespread and not confined to photosynthetic organisms, and structurally and functionally more diverse than chlorophylls. There are currently more than 800 natural carotenoids known, each of which can form several *cis–trans* isomers and be further modified. Chemically, most carotenoids are tetraterpenes: two $C_{20}$-units (originally geranyl-geraniol) are joined tail-to-tail to a chain of 32 carbon atoms bearing eight methyl side chains. Often, this basic carbon $C_{40}$-skeleton is either retained, or only slightly modified, e.g., by cyclization at one or both ends (Figure 3). However, much more extensive modifications are possible, including isomerization and rearrangement of the double bonds, the introduction of oxygen-containing functional groups, and their glycosylation or acylation. This modification seems to be particularly far-reaching in carotenoids dedicated to light harvesting, as exemplified by peridinin; a highly modified $C_{37}$ pigment from algae.

### Spectroscopy

The rod-shaped carotenoids show the typical absorption spectra of linear polyenes, which are characterized by some unusual features. (1) The lowest energy $S_0 > S_1$ transition located in the red to NIR spectral range, is extremely weak (optically forbidden) in most carotenoids, and has been accurately determined only in few pigments by special techniques. (2) The most intense absorption, responsible for the yellow-orange color of most carotenoids, is a series of closely spaced, sometimes overlapping bands in the 400–550 nm range. With an increasing number of conjugated double bonds, this absorption is shifted, in an asymptotic fashion, to the red. The sub-bands are generally well resolved, but can be broadened to a degree that they appear only as a single, somewhat structured band. (2) There is at least one other transition between these two bands, which again is forbidden and therefore very weak. (4) *cis*-Carotenoids show an additional, typical band 100–150 nm to the blue of the main absorption (see Figure 4 for type spectra). Although the "forbidden" bands do not contribute to absorption, they are fundamentally important for the biological functions of carotenoids. The states responsible for them can be reached indirectly, e.g., after absorption into the energy-rich major absorption band and IC, or by energy transfer from neighboring pigments like chlorophylls,

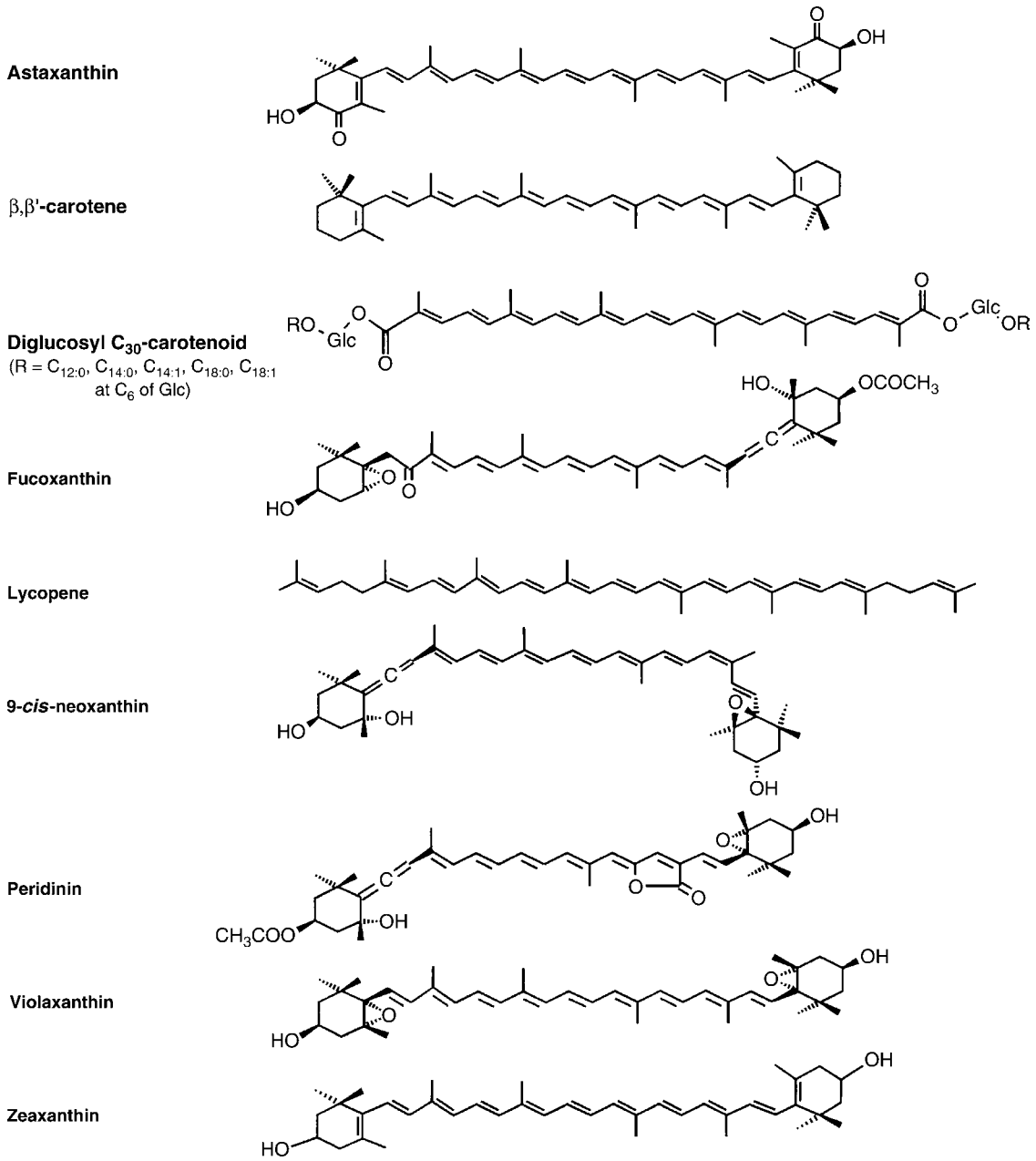



**FIGURE 3** Example of carotenoid structures.

and they thereby contribute to energy transfer and dissipation.

Most carotenoids show extremely rapid IC from all excited states, and therefore negligible fluorescence, with lifetimes in the range of only a few picoseconds. Carotenoids have unusually low-lying triplet states, many of them even below the energy of singlet oxygen (1250 nm). Again, they can be reached only indirectly, mainly via energy transfer from triplets (e.g., of chlorophylls) or from singlet oxygen. Since the latter are highly cytotoxic, this property is functionally very important, too, because it is the basis for the protective effects of carotenoids.

The spectral properties of carotenoids can be modified considerably by interactions with the protein. A spectacular case is the color change of astaxanthin from orange to green when it is bound in the crustacean protein, $\alpha$-crustaxanthin, and its reversion when the protein is denatured by boiling.

## Occurrence and Functions

Carotenoids are almost ubiquitous in living organisms, even though animals including man can not synthesize them but have to rely on dietary supplies.

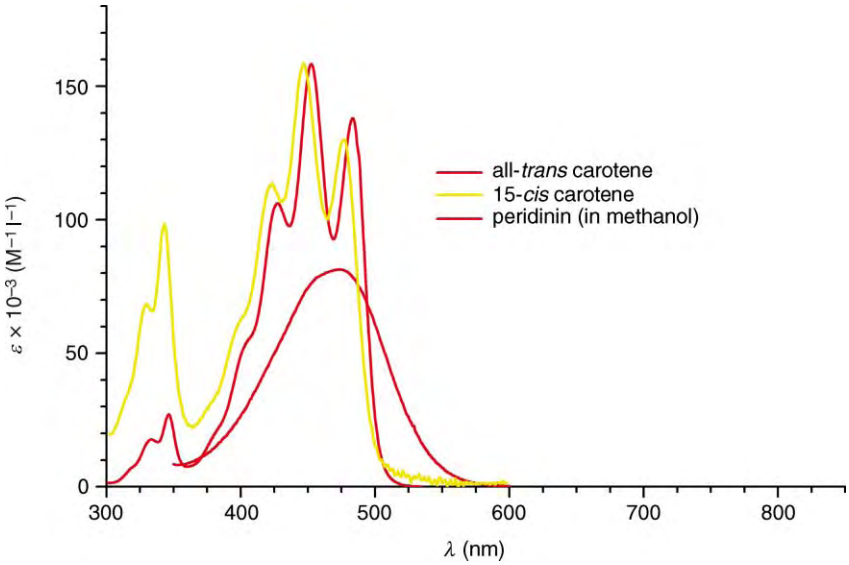



**FIGURE 4** Type spectra of carotenoids.

Their functions are as diverse as their structures. In photosynthesis, their essential function is the protection of the photosynthetic apparatus under conditions of light overload. Whenever the RC cannot follow the energy input from the LHC, the long-lived excited states of the chlorophylls pose a deadly danger to these organisms due the formation of ROS. This is prevented by carotenoids at several levels. They can accept excess energy via their low-lying "forbidden" states, they can quench chlorophyll triplets by triplet energy transfer to the low-lying carotenoid triplets, and they can quench ROS by energy transfer, e.g., from singlet oxygen, or addition of ROS to the double bond system. All these processes require very close distances between donor and acceptor, which is nicely corroborated by X-ray structures of photosynthetic complexes. Specialized carotenoids are found in contact to chlorophylls in the RC and in all chlorophyll-containing LHC. In the latter, they seem to be positioned strategically at critical sites where the energy is funneled to the RC.

Since ROS are also important in the attack of infectious agents by the immune system, bacteria synthesize carotenoids for their protection, and many of the more virulent forms are deeply colored by carotenoids.

Carotenoids also protect from light, simply by their function as light filters, removing NUV and blue light by virtue of their high absorption in these spectral regions, and their capacity to rapidly degrade the excitation energy to heat by IC. Certain algae tolerant to extreme light stress contain droplets of pure carotenoids. And many non-photosynthetic organisms synthesize carotenoids when subjected to increasing light intensities. As nonphototoxic pigments, carotenoids often function as "safe" dyes in nature. Examples are the colors of flowers used for attracting insects, or of crustaceans to blend into the marine background.

In spite of their general protective function, carotenoids have also been adapted by photosynthetic organisms for light harvesting in the "green gap" (470–600 nm) where chlorophylls absorb only poorly, in particular in microalgae and phototrophic bacteria which cannot outgrow their competitors. Two factors are important to this function. One is the development of carotenoids in which the excited state lifetime is somewhat increased, and therefore IC reduced. The two most abundant carotenoids, fucoxanthin and peridinin, show this very nicely with lifetimes reaching more than 100 ps. The second factor is to position the carotenoids in contact with chlorophylls, thereby speeding up energy transfer sufficiently to be effective within the short excited state lifetimes (electron exchange mechanism). Since the direction of energy transfer depends on the relative energies of the excited states, they have to be properly ordered: for light harvesting, the carotenoid excited state needs to be above that of the neighboring chlorophyll; for protection it needs to be below. There is evidence that plants and some algae use, in the so-called violaxanthin cycle, subtle structural modifications in order to manipulate the excited state energies of carotenoids, thereby converting a light harvesting into a protecting pigment, and vice versa, in response to the light supply.

Last but not least are carotenoids precursors for certain important metabolites. Only two examples are stated. (1) Retinal, the chromophore of the visual pigment rhodopsin, is derived from carotene. Since the latter cannot be synthesized by animals, they need it supplied as provitamin A. Retinal derivatives are also required for other regulatory functions.

(2) Certain fragrances of roses have also been shown not to be synthesized directly, but rather to be breakdown products of the flowers' carotenoids.

## Chemical Properties

Due to their hydrocarbon skeleton, almost all are insoluble in water and well soluble in nonpolar solvents. The conjugated double-bond system is chemically moderately stable. It is subject to rearrangements, in particular in the light, and to additions, for example, of oxygen. The chemical properties of the more highly modified carotenoids are determined by their particular functional groups.

## Metabolism

Carotenoids, like all terpenoids, are products of the isopentenyl-pyrophosphate metabolism. This intermediate can be synthesized either by the mevalonate pathway from acetyl-coenzyme A, or via the more recently discovered deoxyxylulose pathway. While still under study, the latter seems to be the pathway leading to carotenoids in most photosynthetic organisms. One of the resulting $C_5$-units (dimethylallyl-pyrophosphate) is condensed sequentially by prenyl transferases in a head-to-tail fashion with three isomeric $C_5$-units (isopentenyl-pyrophosphate) to yield geranylgeranyl-pyrophosphate (GGPP), an important branching point in terpenoid metabolism. GGPP is, for example, directly or indirectly, the substrate for esterifying the C-17 propionic acid side chain of most chlorophylls, thereby linking the tetrapyrrole- and terpenoid pathways. The biosynthesis directed to carotenoids begins with a tail-to-tail condensation of two molecules of GGPP by the dedicated enzyme phytoene synthase. Having only three conjugated double bonds, the resulting phytoene is still uncolored. Dehydrogenation by desaturases via phytofluene to lycopene is common to most carotenoids currently known. $C_{30}$ carotenoids are derived from condensation of two $C_{15}$-units (farnesol-pyrphosphate) in an otherwise very similar reaction sequence. A wide variety of enzymes, many of them still poorly characterized, is responsible for further structural modifications. They include cyclases to form the end rings characteristic of many carotenoids, oxygenases to introduce OH groups, isomerases and (de)hydrogenases to modify the double-bond system, and many more follow-up enzymes allowing for the structural variety of carotenoids.

## Applications

The most frequent applications of carotenoids, which are of considerable economic importance, are as food colorants, as dietary supplies, and as sun screens in cosmetics. Food is frequently colored indirectly, e.g., by supplying carotenoids or carotenoid-rich algae in the feedings of fish (salmon) or poultry (chicken eggs). Provitamin A ($\beta$-carotene) is supplied directly but also indirectly, e.g., as milk supplement. Industry scale synthetic methods have been developed, natural sources are algae such as *Hematococcus*, plants such as carrots, and genetically manipulated bacteria.

## See Also the Following Articles

Chloroplast-Redox Poise and Signaling • Chloroplasts • The Cytochrome $b_6f$ Complex • Light-Harvesting Complex (LHC) I and II: Pigments and Proteins • Photosynthesis • Photosynthetic Carbon Dioxide Fixation

## Glossary

**absorption** The process of interaction of a molecule with a photon to produce an excited state.

**excited states** More precisely, electronically excited states; there are usually generated by light absorption in molecules containing a large number of conjugated double bonds. Singlet and triplet states are distinguished by their lifetimes. If the ground state is a singlet, as in practically all dyes, excited singlet states are short lived ($10^{-12}$–$10^{-8}$ s), and the lowest triplet states are long-lived (up to milliseconds). If the ground state is a triplet, one of the rare cases being molecular oxygen, the lowest excited singlet state is long lived.

**fluorescence** It is the spontaneous decay of an excited state to the ground state with the concomitant emission of the energy in the form of a photon. Together with phosphorescence, which is distinguished from fluorescence mainly by its longer timescale ($>10^{-6}$ versus $<10^{-8}$ s), they are often summarized as luminescence.

**internal conversion** The spontaneous decay of an excited state to the ground state with the concomitant release of the energy in small packages as heat. It is a so-called loss channel, because the heat is (generally) of no use in biology, but is, by the same token, important in light protection, e.g., by preventing photosensitized generation of reactive oxygen species.

**isoprenoids** These are compounds derived from the branched $C_5$-unit, isoprene. Besides carotenoids, important natural isoprenoids are the steroids, gibberellins (plant hormones), mono-, sesqui-, and diterpenes (fragrances and flavors), and polymers like rubber.

**photodynamic therapy (PDT)** This is the diagnostic and therapeutic application, for example in treatment of cancer, which relies on photosensitizing dyes, light, and oxygen to selectively attack cells, virus, tissue, or organs.

**photosensitization** The process of indirectly generating reactive oxygen species by triplet energy transfer. A sensitizing dye is excited by light to the singlet state. This reacts spontaneously to a long-lived triplet state, capable of generating reactive oxygen species by several mechanisms.

**photosynthesis** The natural process of plants, algae and certain bacteria by which (sun)light is converted to biochemical energy (carbohydrates), which is eventually the primary source for life on Earth.

**reactive oxygen species (ROS)** These species comprise several highly aggressive and cytotoxic chemicals derived from oxygen and often produced by action of light, including singlet oxygen, superoxide, peroxide, and hydroxyl radicals.

**tertrapyrroles** These are compounds comprised of four pyrrole rings. In natural tetrapyrroles, they are linked either directly or by single carbon bridges, and they can by linked in a linear (bile pigments) or a cyclic fashion (hemes, chlorophylls, vitamin $B_{12}$).

## Further Reading


Britton, G., Liaaen-Jensen, S., and Pfander, H. (eds.) (1995). *Carotenoids*. Birkhäuser, Basel.

Deisenhofer, J., and Norris, J. R. (eds.) (1993). *The Photosynthetic Reaction Center.* Academic Press, New York.

Frank, H. A., Young, A. J., Britton, G., and Cogdell, R. J. (eds.) (1999). *The Photochemistry of Carotenoids*. Kluwer, Dordrecht.

Green, B., and Parson, W. W. (eds.) (2003). *Light-Harvesting Antennas in Photosynthesis*. Kluwer, Dordrecht.

Hörtensteiner, S., Wüthrich, K. L., Matile, P., Ongania, K., and Kräutler, B. (1998). The key step in chlorophyll breakdown in higher plants. *J. Biol. Chem.* **273**, 15335–15339.

http://photoscience.la.asu.edu/photosyn/photoweb/default.html (photosynthesis at all levels).

http://www.carotenoidsociety.org/ (carotenoids).

http://www.photosynthesisresearch.org (photosynthesis).

http://terra.nasa.gov/Gallery/ (images of the Earth).

Kobayashi, M., Akiyama, M., Watanabe, T., and Kano, H. (1999). Exotic chlorophylls as key components of photosynthesis. *Curr. Top. Plant Biol.* **1**, 17–35.

Eisenreich, W., Rohdich, F., and Bacher, A., (2001). Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant Sci.* **6**, 78–84.

Moser, J. G. (ed.) (1998). *Photodynamic Tumor Therapy: 2nd and 3rd Generation Photosensitizers*. OPA, Amsterdam.

Scheer, H. (ed.) (1991). *Chlorophylls*. CRC Press, Boca Raton, USA.

Vernon, L. P., and Seely, G. R. (eds.) (1966). *The Chlorophylls*. Academic Press, New York.


## Biography

Hugo Scheer studied chemistry in Braunschweig, Germany. After graduation he spent 3 years with J. J. Katz and J. R. Norris at Argonne National Laboratory, USA, and then went in 1975 to the Universität München, Germany, where he became Professor of Plant Biochemistry. His research interest is in chlorophylls and plant biliproteins, with a focus on their functions, on pigment–protein interactions, and on their use as probes in protein dynamics and in photodynamic therapy of cancer.

# Chloroplast Redox Poise and Signaling

**John F. Allen**
*Lund University, Lund, Sweden*

**Chloroplasts are membrane-bound compartments (organelles) in plant and algal cells. Chloroplasts perform all of the component reactions of photosynthesis, including absorption of light energy by chlorophyll; conversion of that energy into chemical potential; electron transfer; synthesis of ATP; and assimilation of carbon dioxide to give carbohydrates. Primary steps in photosynthesis are light-driven redox (reduction–oxidation) reactions; transfer of electrons or hydrogen atoms. These redox reactions function safely and efficiently only within a narrow range of degrees of reduction of their reactants and products. In electron transport pathways, each intermediate must be capable of acting as both an acceptor and a donor, and must therefore be present in both its oxidized and its reduced form. An optimal state of redox poise is achieved by a variety of regulatory mechanisms, operating on different stages of gene expression. Redox signaling occurs when a signal of redox imbalance initiates a change that corrects that imbalance, restoring poise. The response may be within or between cells, and may concern chloroplast or nuclear gene expression. Chloroplast redox signaling utilizes the chloroplast genome and apparatus of gene expression. Redox signaling may require, and be the reason for, both chloroplast and mitochondrial genomes.**

## The Redox Chemistry of Photosynthesis

### Redox Reactions and Photosynthesis

A redox reaction involves an electron or hydrogen atom donor, and an electron or hydrogen atom acceptor. During the course of the redox reaction, as the electron or hydrogen atom is transferred, the donor reduces the acceptor, and the acceptor oxidizes the donor. Thus, an electron (or hydrogen) donor is a chemical reductant, and an electron (or hydrogen) acceptor is a chemical oxidant. The direction of electron (or hydrogen) transfer between two chemical species is determined by their relative activities and also by their relative electrochemical potentials, or redox potentials, as given by the Nernst equation. A species with a lower redox potential will tend to act as a reductant, or donor, when coupled in a redox reaction with a species with a higher redox potential, acting as the oxidant, or acceptor.

In photosynthesis, light-driven electron transport may be cyclic, or linear (also known as "noncyclic"). Linear electron transport requires an electron donor and an electron acceptor. In many bacteria the donor is an organic compound, or an inorganic reductant such as $H_2S$ or $H_2$. In cyanobacteria and chloroplasts, the donor is water, and oxygen is released as a by-product. The electron acceptor is $NADP^+$. Together with ATP produced by photosynthetic phosphorylation, NADPH represents stored energy, which may be used to drive carbon dioxide assimilation and processes including synthesis (e.g., protein synthesis) and transport.

### van Niel and Bacteria

Photosynthesis occurs in bacteria, and in most plants and algae. Even in eukaryotes, photosynthesis is localized in chloroplasts, which are bacterial in origin. The microbiologist Cornelius van Niel, working in Stanford, California, showed that photosynthetic bacteria use light as a source of energy to cause hydrogen atom transfer to carbon dioxide, from any one of a range of weak reducing agents, collectively designated "$H_2A$" in a reaction described by the van Niel equation:

$$2H_2A + CO_2 \xrightarrow{\text{light}} CH_2O + 2A + H_2O$$

In the special case of oxygenic photosynthesis in plant and algal chloroplasts and in cyanobacteria, the weak reducing agent ($H_2A$) is water ($H_2O$), and the van Niel equation then becomes:

$$2H_2O + CO_2 \xrightarrow{\text{light}} CH_2O + O_2 + H_2O$$

### The Hill Reaction

Independently, Robert (Robin) Hill, in Cambridge, showed that chloroplasts isolated from leaves

 © 2004, Elsevier Inc. All Rights Reserved. 

produce oxygen in the light, provided a suitable electron acceptor, or "Hill oxidant" is available ("A," below):

$$2H_2O + 2A \xrightarrow{\text{light, chloroplasts}} 2AH_2 + O_2$$

Assimilation of $CO_2$ by reoxidation of $AH_2$ will give "complete" photosynthesis, and its overall formulation is identical to a simplified version of the oxygenic version of the van Niel equation:

$$H_2O + CO_2 \xrightarrow{\text{light, chloroplasts}} CH_2O + O_2$$

## Reaction Centers

How does light drive electron or hydrogen atom transfer? Louis Duysens, in Leiden, and others demonstrated light-induced oxidation of chlorophyll (Chl) at photosynthetic reaction centers, and it became clear that some chlorophyll molecules themselves undergo photo-oxidation after absorbing a quantum of light and reaching an excited state ($Chl^*$). Photosynthetic electron transport is initiated when the chlorophyll excited state decays to a ground state by passing an electron on to an acceptor (A), and by taking one from a donor (D):

$$DChlA \xrightarrow{\text{light}} DChl^*A \rightarrow DChl^+A^- \rightarrow D^+ChlA^-$$

## The Z-Scheme and Two Photosystems

In 1960, Robert Hill and Fay Bendall proposed a "Z-scheme" for photosynthetic electron transport, in which two separate photosystems, termed I and II, each with a core reaction center, act as intermediates in the chloroplast electron transport chain. The two photosystems are connected, in series, by electron carriers (Figure 1).

## Chloroplast Function

Intrinsic to internal membranes called thylakoids, chloroplasts contain chlorophyll and other pigments (such as carotenoids) that harvest and convert light energy, an electron transport chain that intersects with light-harvesting pigments at photosynthetic reaction centers, and an ATPase that couples electron transport with synthesis of ATP. The soluble phase of the chloroplast usually contains the enzymes and intermediates of the Benson–Calvin cycle of assimilation of carbon dioxide. In development, chloroplasts originate from plastids in parallel with nonphotosynthetic plastids with other specific functions in metabolism. Although most plastid proteins are imported from the cytosol, as precursors, all plastids also contain DNA, RNA, ribosomes, and a genetic system, which is responsible for synthesis of some of their components.

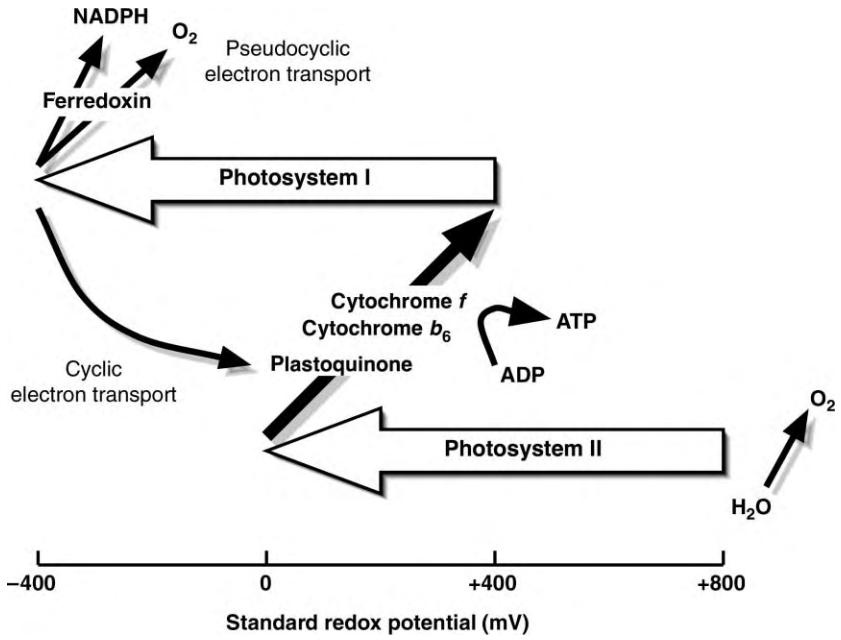



**FIGURE 1** The two-light reaction model, or "Z-scheme," for photosynthetic, noncyclic electron transport and photophosphorylation (ATP synthesis) in chloroplasts. Noncyclic electron transport involves photosystems I and II, which are connected in series. The cyclic and pseudocyclic pathways are superimposed on the noncyclic pathway.

Chloroplasts, in particular, still bear a striking resemblance to prokaryotic cyanobacteria, from which all plastids evolved.

### Photophosphorylation

In 1954, F. R. (Bob) Whatley, Mary Belle Allen, and Daniel I. Arnon, in Berkeley, California showed that isolated chloroplasts carry out ATP synthesis in the light, a process also demonstrated for isolated bacterial membranes by Albert Frenkel:

$$ADP + P_i \xrightarrow{\text{light, chloroplasts}} ATP + H_2O$$

#### Cyclic

The original chloroplast photophosphorylation of Whatley, Allen, and Arnon was not accompanied by net redox changes, and is coupled to cyclic electron transport. In the Z-scheme, this cyclic electron transport drives cyclic photophosphorylation, and requires photosystem I alone (Figure 1), although photosystem II activity and other factors greatly affect its onset.

#### Noncyclic

The Arnon group later showed that ATP synthesis is also coupled to the Hill reaction, and the yield of ATP occurs in a fixed stoichiometry with reduction of a Hill oxidant. There is thus a distinction between cyclic and noncyclic photophosphorylation. After 1960 it was realized that noncyclic photophosphorylation normally requires both photosystems I and II (Figure 1).

#### Pseudocyclic

In 1952, Alan Mehler discovered that molecular oxygen will act as a Hill oxidant. Noncyclic electron transport (Figure 1) with oxygen as the terminal acceptor is coupled to ATP synthesis, and requires photosystems I and II. However, oxygen is both consumed and produced in this reaction, no net oxidation–reduction is observed, and so the ATP synthesis resembles cyclic one, and is known as pseudocyclic phosphorylation.

## Kinetics and Maintenance of Redox Poise

The interplay between noncyclic, pseudocyclic and true cyclic photosphosphorylation first gave rise to the concept of "redox poise" in chloroplast photosynthesis. If the photosystem I of the Z-scheme (Figure 1) is the same for all three forms of phosphorylation, then there is competition between paths of electron transport.

An optimal state for cyclic electron tranport is one of maximal "redox poise," when the total pool of each component gives a redox state that is 50% oxidized and 50% reduced. The rapid, primary photochemical reactions at photosynthetic reaction centers use light energy to move electrons in the direction opposite to that predicted by their redox potentials: the donor has a much higher redox potential than the acceptor, and light is therefore used to generate a redox couple that is far from redox equilibrium. Secondary electron transport nevertheless moves the electrons in the predicted direction, towards equilibrium. "Redox poise" can then still be applied to linear or noncyclic electron transport, and especially to any of its components. When applied to noncyclic electron transport, "redox poise" indicates a position of optimal redox state where the activities of components are such that their effective redox potentials favor physiologically useful electron transfer. Two extreme departures from redox poise exist in the form of states of over-reduction and over-oxidation (Figure 1). A cyclic chain is said to be over-reduced when all components are in their reduced forms; there are no electron acceptors. The same chain is said to be overoxidized when all components are in their oxidized forms; there are no electrons to cycle. When applied to noncyclic electron transport, "over-reduced" and "overoxidized" can be applied to indicate the preponderence of one or other redox state, giving the tendency of a component to engage in redox chemistry with donors or acceptors that are presumed to be nonphysiological, especially where reactive and toxic chemical species are produced in consequence. Oxygen, for example, readily accepts electrons from most components of the photosynthetic chain, a product is the superoxide anion radical, and redox poise may ensure that the "correct" electron transfer competes kinetically with reduction of oxygen.

### Cyclic Electron Transport and Photophosphorylation

As first identified by Whatley and Bruce Grant, the onset of cyclic photophosphorylation is delayed, sometimes indefinitely, under anaerobic conditions. The delay can be avoided by addition of an inhibitor of electron transport in photosystem II, or by choice of a wavelength of illumination that is selective for photosystem I. It was concluded that the cyclic chain can become over-reduced when photosystem II is active, and no electron acceptor is available. Over-reduction could also be reversed, and redox poise restored, if a pulse of an oxidant, such as potassium ferricyanide or oxygen, is added to remove excess electrons from the cyclic chain.

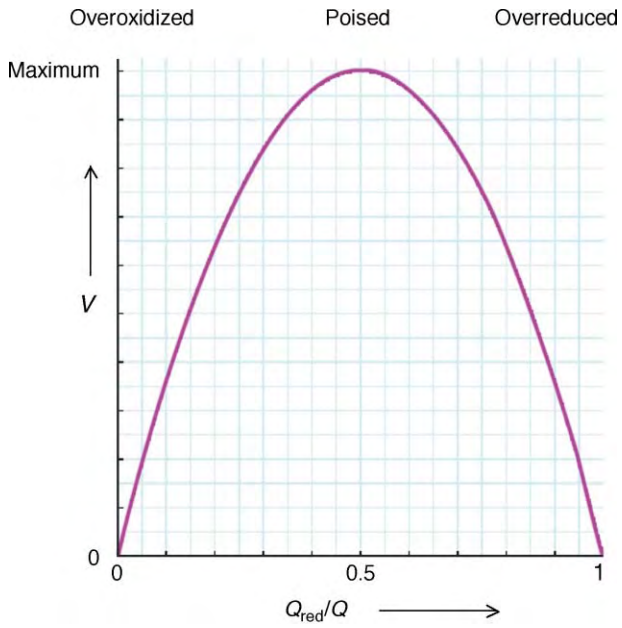



**FIGURE 2** The ideal rate, $v$, of cyclic electron transport as a function of the redox ratio of one of its components, Q. Q must be present in both its oxidized and reduced forms: in cyclic electron flow, Q must be both a donor and acceptor of electrons.

In contrast, under aerobic conditions, Grant and Whatley found that the photosystem II inhibitor, just like a choice of wavelength specific to photosystem I, actually inhibits photosystem I-cyclic photophosphorylation. This is explained by the ability of oxygen to accept electrons from photosystem I, so that, in the absence of photosystem II activity, the cyclic chain becomes drained of electrons, or overoxidized (see Figure 2).

### Photosystem 1 Cyclic Electron Flow

The contribution of cyclic photophosphorylation to photosynthesis (Figure 1) depends on maintaining redox poise, and avoiding over-reduction and overoxidation (Figure 2). There is evidence that oxygen acts as a poising oxidant under physiological conditions. Ulrich Heber and colleagues in Wurzburg showed that intact, functional chloroplasts are held in an indefinite lag phase under anaerobic conditions. However, if a pulse of oxygen is given at any time, sufficient ATP is produced to start the Benson–Calvin pathway, and regeneration of the electron acceptor $NADP^+$ then allows whole-chain electron transport to generate oxygen, maintaining a poised cyclic chain, ATP synthesis, and complete photosynthesis. *In vivo*, down-regulation of photosystem II may also counteract over-reduction, and chloroplast NAD(P)H dehydrogenase activity may counteract overoxidation.

### The Q-Cycle

In the electron transport chain between photosystems I and II, the cytochrome $b_6f$ complex catalyzes a cycle of electrons through two cytochromes $b$, involving intermediate states of the two-electron carrier plastoquinone. Redox poise is likely to be essential in the Q-cycle, where the plastosemiquinone participates in one-electron transfer with cytochrome $b$, despite its tendency to transfer its single electron to oxygen, generating superoxide.

### Noncyclic Electron Transport

In intact chloroplasts, continued operation of noncyclic electron transport seems to depend on production of some additional ATP by cyclic electron transport. The ATP is required to drive the Benson–Calvin cycle, and therefore for regeneration of the physiological electron acceptor, $NADP^+$. Insufficient ATP therefore "stalls" photosynthesis by causing over-reduction of the cyclic chain.

### Oxygen and Poise

Oxygen is an effective electron acceptor for noncyclic electron transport, and also acts as a poising oxidant for cyclic electron transport. However, the products of oxygen reduction are superoxide and hydrogen peroxide. Reduction of oxygen by the iron–sulfur protein and electron transport intermediate, ferredoxin, may proceed by a physiologically safe route of two one-electron transfers, and also lead to creation of "antioxidant" defenses against the toxicity of reactive oxygen species.

## Posttranslational Modification of Pre-Existing Proteins

Regulatory devices that maintain redox poise also extend to gene expression at different levels. Feedback control loops involving components of electron transfer chains and stages in gene expression are seen in all bioenergetic systems. Close association between bioenergetic coupling membranes and genes for their protein components is conspicuous in chloroplasts and mitochondria. The need for direct redox signaling has been proposed as the primary reason for the persistence, in evolution, of chloroplast and mitochondrial genomes.

### State Transitions

Control of gene expression by posttranslational modification underpins a well-known process, namely, state transitions. Photosystems I and II have different

light-harvesting pigment systems, and different absorption and action spectra. Thus, any randomly chosen wavelength of light is likely to favor either photosystem I or photosytem II. Yet their connection in series requires a fixed ratio of rates of electron transport through their reaction centers, 1:1 for noncyclic electron transport alone, but incrementally more (estimated at 20%) to photosystem I to account for additional, cyclic electron transport. Cecilia Bonaventura and Jack Myers in Austin, Texas, and Norio Murata in Tokyo independently showed that different unicellular algae redistribute absorbed excitation energy between photosystems I and II, as if to achieve balanced delivery of energy to the two reaction centers. Light delivered to one reaction center whose rate is limited by another will be wasted as heat or fluorescence. Redistribution therefore achieves maximal efficiency despite changing wavelengths of light that otherwise favor one photosystem or the other. Photosystem II works effectively at wavelengths up to ~660–670 nm; photosystem I can utilize light beyond this "red drop" in photosynthetic yield. A beam of light with a spectrum centered at, say, 700 nm, will be selective for photosystem I, and is termed "light 1." A beam centered below 660 nm will drive both photosystems, but is required for photosystem II, and is termed "light 2." Switching beams from light 2 to light 1, or superimposing light 1 onto light 2, induces a change in the light-harvesting apparatus, redistributing energy to photosystem II at the expense of photosystem I. The state arrived at is called the "light 1 state" or "state 1." Conversely, switching from light 1 to light 2 induces a "light 2 state," or "state 2."

### Chloroplast Protein Phosphorylation

John Bennett at Warwick University discovered chloroplast protein phosphorylation and showed that one conspicuous phosphoprotein was a light-harvesting chlorophyll *a*/*b*-binding protein forming part of chloroplast light-harvesting complex II (LHC II). LHC II is an intrinsic membrane protein which becomes phosphorylated on a threonine residue by the action of a membrane-associated LHC II kinase.

### Plastoquinone Redox Control of the LHC II Kinase

The LHC II kinase was originally thought to be activated by light. However, in Urbana, Illinois, in a collaboration with Bennett, John F. Allen and Charles J. Arntzen showed that LHC II becomes phosphorylated in darkness, provided the electron carrier plastoquinone is chemically reduced. The apparent light activation is sufficiently explained as redox-activation, with electrons being supplied to plastoquinone from photosystem II. In addition, LHC II phosphorylation is accompanied by changes in chlorophyll fluorescence and in electron transport, demonstrating that absorbed light energy simultaneously became redistributed to photosystem I at the expense of photosystem II.

### Distribution of Absorbed Excitation Energy

The state-2 transition, an apparently purposeful response increasing efficiency of energy conversion, is the result of activation of the LHC II kinase by reduced plastoquinone. In light 1, the state-1 transition results from oxidation of plastoquinone by photosystem I: the LHC II kinase becomes inactivated, a light- and redox-dependent phospho-LHC II phosphatase catalyzes dephosphorylation of phospho-LHC II, and light energy absorbed by chlorophyll molecules of LHC II is returned to photosystem II, at the expense of photosystem I (Figure 3). Phosphorylation affects the three-dimensional structure of LHC II, and the movement of LHC II between photosystems I and II proceeds because its two structural forms differ in their capacity to bind and interact functionally with the two photosystems – a change in molecular recognition. An LHC II kinase has been identified by Jean–David Rochaix and co-workers in Geneva.

### Thioredoxin

A number of enzymes of the Benson–Calvin cycle, and the coupling ATPase of chloroplast membranes, are activated by the reduced form of a soluble iron–sulfur protein, thioredoxin. This example of chloroplast redox signaling was discovered by Robert (Bob) Buchanan and co-workers in Berkeley, California. Thioredoxin accepts electron from ferredoxin, an electron carrier on the acceptor side of photosystem I, and light activation therefore depends on the activity of both photosystems. The function of thioredoxin redox signaling seems to be to ensure that $CO_2$ assimilation and ATP synthesis are inactivated in darkness, so that energy stored in photosynthesis is not subsequently dissipated.

## Posttranscriptional Control

Within chloroplasts, reduced thioredoxin also activates ribosomal translation and processing of RNA, and has been studied for the *psbA* gene product (the D1 reaction center apoprotein of photosystem II) by Steven Mayfield at the Scripps Institute in San Diego, California.

## Transcriptional Control

Redox and other signal transduction pathways in bacteria often exert parallel effects at posttranslational

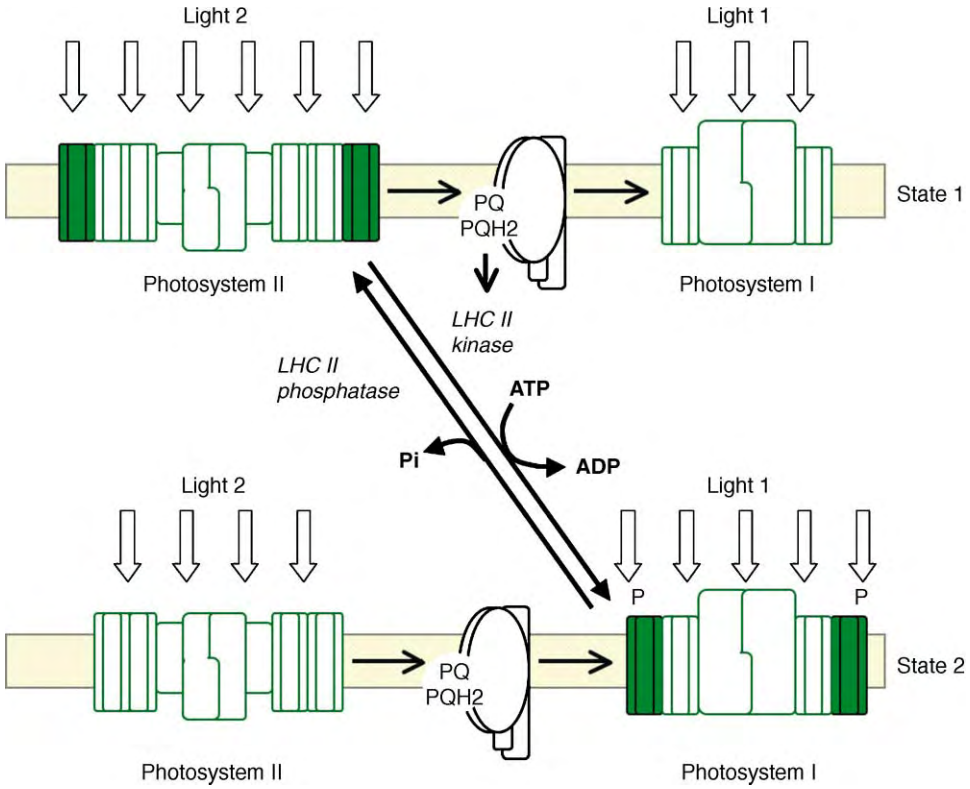



**FIGURE 3** Plastoquinone redox control of LHC II kinase activity and its role in balancing distribution of excitation energy between photosystems I and II.

and transcriptional levels of gene expression. Chloroplasts are no exception.

### Chloroplast Genes

Thomas Pfannschmidt and John F. Allen, working at Lund University, showed that transcription of chloroplast genes for reaction center apoproteins is regulated by the redox state of plastoquinone. As with phosphorylation of LHC II, the direction of control is functionally intelligible. Reduced plastoquinone is a signal that photosystem I is rate-limiting and photosystem II activity is in excess. Reduction of plastoquinone turns off transcription of photosystem II reaction center genes, and turns on transcription of photosystem I reaction center genes. Conversely, oxidized plastoquinone is a signal of imbalance with photosystem II rate-limiting, and photosystem I in excess: oxidized plastoquinone turns off photosystem I transcription, and turns on photosystem II transcription. Thus, the stoichiometry of photosystems I and II will tend to adjust itself to match changes in the prevailing light regime, as well as changes in metabolic demand for ATP relative to NADPH. Plastoquinone redox control of reaction center gene transcription maintains redox poise of plastoquinone (Figure 4) and of other components linked to it, including components of the cyclic electron transport pathway of photosystem I (Figure 1).

### Nuclear Genes

Redox signals from the chloroplast also affect transcription of nuclear genes. In the case of light 1–light 2 effects and redox control at the level of plastoquinone, nuclear genes for photosystems I and II are regulated in the same functionally intelligible way, suggesting a backup which serves the same goal of maintaining redox poise within the chloroplast.

## Mechanisms and Evolutionary Implications

Mechanisms and components in chloroplast redox signaling are not fully resolved, especially as regards transcription and translation. Based on bacterial redox signaling, candidate pathways are proposed as follows, and preliminary evidence is consistent with these.

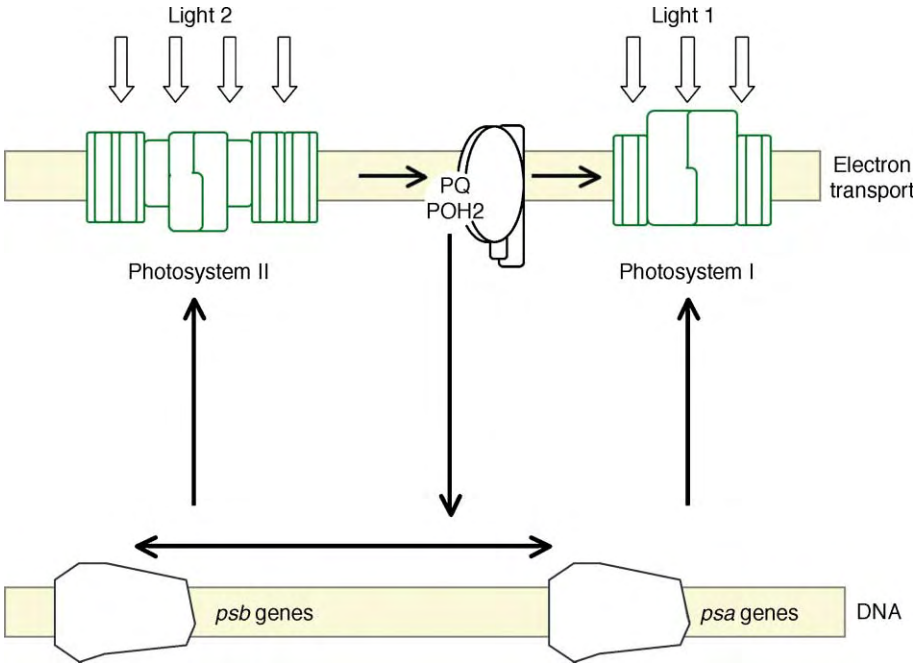



**FIGURE 4** Plastoquinone redox control of chloroplast photosynthetic reaction center gene transcription and its role in adjusting the stoichiometry of photosystem I to photosystem II.

## Two-Component Systems

A bacterial two-component redox regulatory pathway utilizes a membrane-intrinsic histidine sensor kinase which becomes phosphorylated when ubiquinone in the respiratory chain is reduced. The phosphate from the sensor is then transferred to an aspartate of a soluble response regulator. The response regulator is a sequence-specific DNA-binding protein, and the effect is to switch on transcription of genes for proteins required for anaerobic respiration. This aerobic respiratory control (Arc) system senses redox state. Photosynthetic bacteria, including cyanobacteria, possess cognate systems. Chloroplast genomes of some eukaryotic, red and brown algae have genes for similar components (sensors and response regulators), and green algae and plants have nuclear genes for these components, including transit sequences required for chloroplast import.

## FNR and Redox Activators

At the level of thioredoxin, accepting electrons from ferredoxins, bacteria have DNA-binding iron–sulfur proteins. The best-studied example is "fumarate and nitrate reductase" of *Escherichia coli* (FNR: by coincidence, the abbreviation also for ferredoxin–NADP reductase of photosystem I). Since reduced ferredoxin sends redox signals to gene expression, it is likely that a cognate redox activator system is involved.

## Redox Signaling and the Function of Cytoplasmic Genomes

Redox signaling is unique neither to chloroplasts nor to photosynthesis. Close coupling between primary redox chemistry and gene expression is seen in bacteria and chloroplasts, with some preliminary evidence also in mitochondria. Inspection of the genes contained in chloroplast and mitochondrial DNA reveals no clear correlation with the hydrophobicity of the gene product, as sometimes supposed. Rather, the core, membrane-intrinsic proteins of primary electron transfer are always encoded in chloroplasts and mitochondria, and synthesized on organelle ribosomes. This rule is especially clear for chloroplast reaction center genes, whose transcription is known to be controlled by the redox state of the plastoquinone pool. It has been proposed that retention, in evolution, of chloroplast and mitochondrial genetic systems gives a co-location of gene with gene product, and that this co-location is essential for redox regulation. Chloroplast redox poise and signaling therefore have important implications for cell evolution, and may account for the distribution of genes between nucleus and cytoplasm in eukaryotic cells.

## See Also the Following Articles

Chlorophylls and Carotenoids • Chloroplasts • Ferredoxin • Ferredoxin-NADP$^+$ Reductase • Photosynthesis

## Glossary

**chloroplast** (from Greek for "green box") A subcellular organelle of plants and algae. Chloroplasts are the location for the process of photosynthesis in eukaryotes.

**photosynthesis** The capture of light energy and its utilization to produce reduced carbon compounds whose subsequent oxidation in respiration releases the free energy originally obtained from light.

**poise** A state of balance. "Redox poise" in electron transport occurs when each electron-carrying intermediate is present in both its oxidized state and its reduced state, in order for that component both to accept and to donate electrons or hydrogen atoms.

**redox** An adjective derived from "reduction–oxidation," and describing a class of chemical reactions that involve transfer of electrons or hydrogen atoms.

**redox signaling** Coupling between biological electron transfer and gene expression. Regulatory control is exerted in both directions.

## Further Reading

Allen, J. F. (1992). Protein phosphorylation in regulation of photosynthesis. *Biochim. Biophys. Acta* **1098**(3), 275–335.

Allen, J. F. (2002). Photosynthesis of ATP-electrons, proton pumps, rotors, and poise. *Cell* **110**(3), 273–276.

Allen, J. F. (2003). The function of genomes in bioenergetic organelles. *Philos. Trans. Roy. Soc. London Series B-Biological Sci.* **358**(1429), 19–38.

Allen, J. F. (2004). Chromosome b6f: Structure for signalling and vectorial metabolism. *Trends Plant Sci.* **9**(3), 130–137.

Bauer, C. E., Elsen, S., and Bird, T. H. (1999). Mechanisms for redox control of gene expression. *Annu. Rev. Microbiol.* **53**, 495–523.

Blankenship, R. E. (2002). *Molecular Mechanisms of Photosynthesis*. Blackwell Science, Oxford.

Heathcote, P., Fyfe, P. K. and Jones, M. R. (2002). Reaction centres: The structure and evolution of biological solar power. *Trends Biochem. Sci.* **27**(2), 79–87.

Pfannschmidt, T. (2003). Chloroplast redox signals: How photosynthesis controls its own genes. *Trends Plant Sci.* **8**(1), 33–41.

Race, H. L., Herrmann, R. G. and Martin, W. (1999). Why have organelles retained genomes? *Trends Genet.* **15**(9), 364–370.

Wollman, F. A. (2001). State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. *EMBO J.* **20**(14), 3623–3630.

## Biography

John F. Allen is Professor of Plant Cell Biology in Lund University, Sweden. He was educated in Newport, Monmouthshire, UK, and then at King's College in London University, obtaining his Ph.D. with David O. Hall. Dr. Allen carried out postdoctoral work in Oxford and Warwick Universities and in the University of Illinois at Urbana, subsequently working in Leeds University, and, on sabbatical, in University of California, Berkeley. He was Professor of Plant Physiology in Oslo for two years before moving to his present position in 1992. Allen demonstrated superoxide production by isolated chloroplasts and, later, plastoquinone redox control of chloroplast protein phosphorylation. His work on redox regulation of photosynthesis contributed to his theory that chloroplast and mitochondrial genomes allow genetic control of redox poise.

# Chloroplasts

**Nicoletta Rascio**
*University of Padova, Padova, Italy*

**Chloroplasts are the organelles specialized in carrying out the photosynthetic process, which uses light energy to synthesize organic compounds: for this reason they are common to all photoautotrophic eukaryotes.**

**Besides the biosynthetic pathways directly related to photosynthesis, such as synthesis of pigments (chlorophylls and carotenoids), conversion of $CO_2$ to carbohydrates and reduction and organization of sulfur and nitrogen, several other metabolic pathways occur in chloroplasts. These organelles produce or participate in the production of a series of essential compounds required by other cell compartments. For instance, they are the primary site of biosynthesis of fatty acids, isoprenoids, tetrapyrroles, and aminoacids, as well as of purines, pirimidines, and pentoses necessary for nucleic acid build-up. Thus, chloroplasts, in addition to photosynthesis, play other essential roles in sustaining the metabolism of the cell and the whole plant. The complexity and variety of chloroplast activities can arise from the fact that this, which is now a cell organelle, was originally an organism.**

## Chloroplast Origin

The chloroplast originated from a cyanobacterium-like prokaryotic ancestor with oxygenic photosynthesis, which was engulfed by a heterotroph proeukaryote. This endosymbiotic event, which took place about 1 billion years ago, was of enormous importance, since it triggered the evolution of photosynthetic eukaryotes and even led, as a consequence, to the expansion of the heterotroph forms of life on the Earth.

During the evolutionary route which changed it into the chloroplast, the cyanobacterium-like ancestor underwent substantial modifications. A significant event was the transfer of most prokaryotic DNA to the host cell nucleus. The genome of a present chloroplast, actually, codes for ~100 proteins, while the DNA of a free-living cyanobacterium, like *Synecocystis*, whose entire sequence is known, encodes more than 3000 proteins. The massive "endosymbiotic gene transfer" possibly occurred to avoid the mutagenic load for organelle genes due to free radicals produced by photosynthetic light reactions, and to escape the accumulation of deleterious mutations caused by the genetic isolation of the organelle DNA in the host cells (the so-called Muller's ratchet).

This transfer of genetic information made it necessary for the chloroplast to recover the proteins encoded in the nucleus and this was achieved by inserting in the nuclear genes a presequence coding for a transit peptide which targeted the choroplast proteins and redirected them to the organelle.

Another essential change undergone by the evolving chloroplast concerned the photosynthetic light-harvesting pigments. With the exception of some algal groups (Rhodophytes and Cryptophytes), the original phycobiliproteins were replaced by chlorophyll forms, in particular by chlorophyll *b* in green organisms.

## Chloroplast Organization

Algae and lower plants have chloroplasts which can vary considerably in shape and size, whereas in higher plants these organelles are commonly lens-shaped and measure ~5–10 μm.

### Envelope

The chloroplast (Figure 1) is enclosed by a pair of concentric membranes forming the "envelope." The inner envelope membrane is highly selective and contains numerous carriers which regulate the flows of metabolites and ions between chloroplast and the cytosol. The most abundant is the phosphate translocator, which plays a major role in supplying the cell with the products of photosynthesis. The inner membrane also supports some enzymes of biosynthetic pathways, like those of carotenoids, tetrapyrroles, and fatty acids. The outer envelope membrane contains a nonspecific porine-like protein, which allows ions and metabolites of up to ~10 kDa to pass. It is essentially involved in recognizing the cytosol-synthesized chloroplastic proteins which must be transferred into the organelle. This translocation occurs through two multiproteic complexes, one of them inserted in the outer membrane (translocation outer complex, TOC), the other in the inner membrane (translocation inner complex, TIC) of the envelope.

 © 2004, Elsevier Inc. All Rights Reserved. 

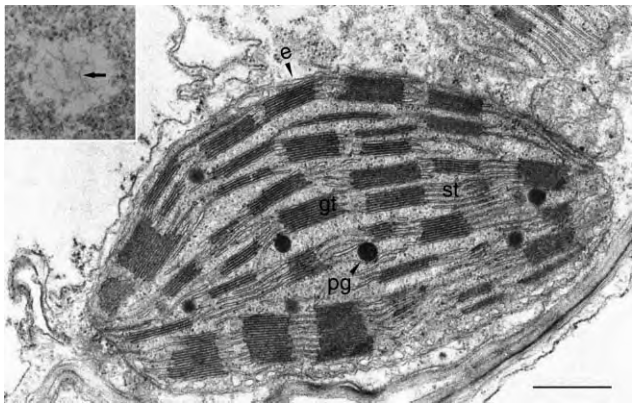



**FIGURE 1** Transmission electron microscope (TEM) micrograph of a chloroplast from a mesophyll cell in a maize leaf. Note the envelope (e) the inner membrane system formed by stacked granal thylakoids (gt) and single stromal thylakoids (st). Some plastoglobules (pg) are present in the stroma which look finely granular due to the presence of 70S ribosomes. (bar = 1 μm). The insert shows the region of a chloroplast stroma with DNA microfibrils (arrow).

## Stroma

The chloroplast internal milieu, called "stroma", looks finely granular due to the presence of 70S ribosomes. It contains prokaryotic DNA (Figure 1), RNA and the entire protein synthesis machinery allowing the organelle to translate its own genetic information. In the stroma are also inserted the soluble enzymes engaged in the numerous biosynthetic pathways. The ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), which catalyzes $CO_2$ fixation in the first reaction of the Calvin–Benson cycle, is the most abundant enzyme, and is even the most abundant protein in the world. Small roundish inclusions, named plastoglobules, made up of plastoquinones, carotenoids, and proteins, are common in the stroma, where also starch grains of photosynthetic origin can occasionally accumulate.

## Thylakoid System

Within the chloroplast a membrane system, the "thylakoid system", is present, which contains the multiproteic complexes involved in the light reactions of photosynthesis. The inner membrane system of the chloroplast (Figure 1) consists of coupled lamellae (thylakoid membranes) separated by a narrow intermembrane space. They form flattened cisternae or saccules (thylakoids), intercommunicating to constitute a continuous and closed membrane system, whose internal space (lumen) is totally isolated from the stromal environment.

A feature common to the green plant chloroplasts (Figures 2A and 2B) is the stacking of thylakoids (granal thylakoids) one upon the other to make piles of saccules named "grana" (singular: granum), which are interconnected by single unstacked thylakoids running in the stroma (stromal thylakoids). This kind of organization, which gives rise to a huge surface area of thylakoid membranes, acquires functional significance, taking into account that, as stated above, these membranes support the complexes carrying out the photosynthetic light reactions and the correlated electron transport and ATP synthesis (precisely: photosystem I (PSI), photosystem II (PSII), the cytochrome $b_6f$ complex (Cyt $b_6f$), and the ATP synthase). These components are diversely and specifically located in the thylakoid membranes (Figure 3). Most of PSII resides in the approached regions of stacked thylakoids (partitions), while PSI and the ATP synthase only occur in the stromal thylakoids and in all the unstacked regions of granal thylakoids (end granal membranes and margins). Finally, the Cyt $b_6f$ is rather homogeneously distributed in the thylakoid system.

A peculiarity of chloroplast membranes, which distinguishes them from the other cell membranes, is the great abundance of glycolipids, represented by two neutral galactolipids, the monogalactosyldiacylglycerol

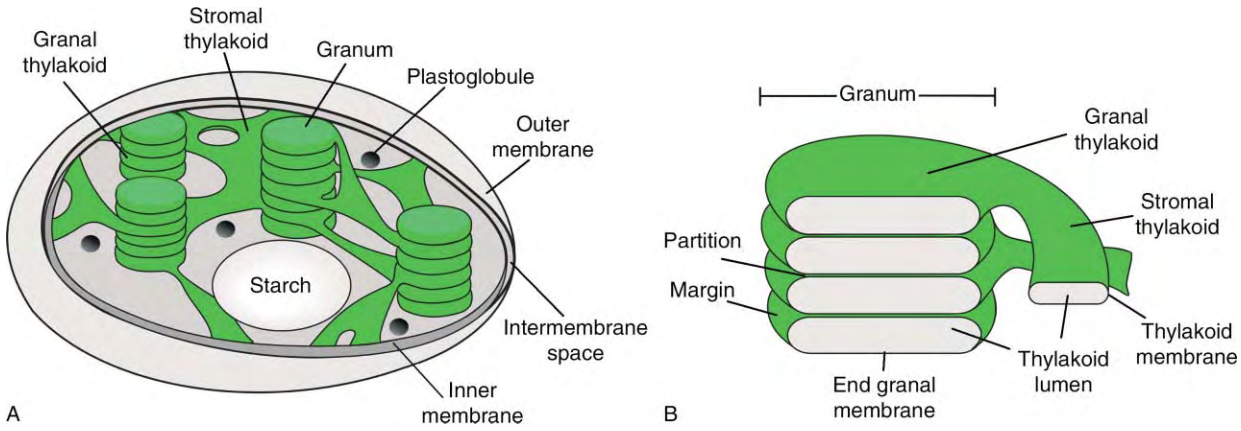



**FIGURE 2** (A) Three-dimensional scheme of a chloroplast. (B) Particulars of the thylakoid system organization.

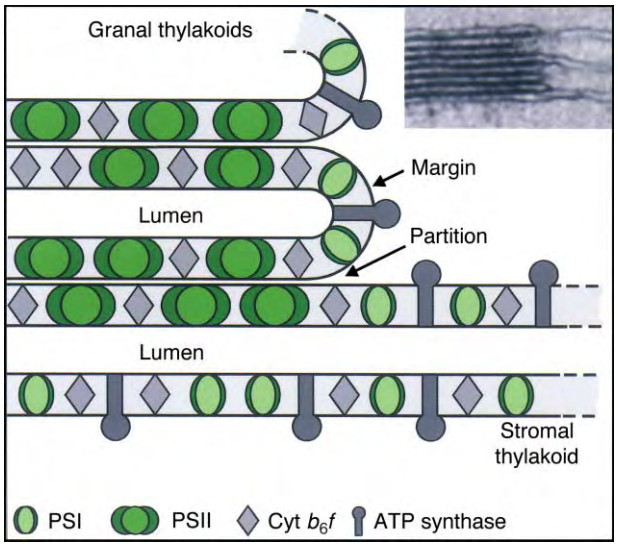



**FIGURE 3** Distribution of the multiproteic complexes in the thylakoid membranes.

(MGDG) and the digalactosyldiacylglycerol (DGDG), which account for 70% of the entire acyl-lipid moiety, and by the anionic sulfolipid sulfoquinovosildyacylglycerol (SQDG). Phosphatidylglycerol (PG) is the only phospholipid present in the membranes. A feature of all thylakoid lipids is the high concentration of polyunsaturated fatty acids, which maintain membrane fluidity in spite of the high protein/lipid ratio. Nonpolar lipids, like plastoquinones, phylloquinones, and tocopherols are inserted in the hydrophobic region of the membrane matrix. Plastoquinone-9, a component of the photosynthetic electron transport chain, is particularly abundant.

## Chloroplast Biogenesis

In higher plants chloroplasts develop from proplastids, which are small undifferentiated organelles present in the meristematic cells. Chloroplast differentiation involves the synthesis of pigments, proteins, and lipids and the structural and functional organization of the whole thylakoid system, as well as the acquisition of the enzymatic components carrying out the numerous metabolic pathways of the mature organelle. These events are accompanied by the increase in the developing chloroplast size and the rise of the organelle number in the cells. Proplastids and young chloroplasts, indeed, can divide through a process of "binary fission" similar to that occurring in bacteria. The chloroplast division is independent of cell division, being part of the developmental program which defines the final number of photosynthetic organelles in the mature green cell.

The dependence on both nuclear and own genetic information makes chloroplast biogenesis a quite complex process requiring the coordinated expression of genes located in the two cellular compartments. This takes place through environmental cues, the most important of which is light, and through endogenous nucleus–chloroplast signaling. The nucleus exerts a preeminent role in controlling the chloroplast gene expression. However, also chloroplast-to-nucleus signaling pathways exist that greatly affect the transcription of nuclear genes encoding photosynthesis-related proteins. This kind of "retrograde" informational flow is triggered by different chloroplast-generated signals, involving, for instance, chlorophyll precursors or the plastoquinone redox state, and serves to correlate the nuclear gene expression with the chloroplast developmental stage and the organelle functionality, but also to achieve a swift photosystem adjustment in response to qualitative and quantitative light changes in the plant growth environment.

## Light and Chloroplast Differentiation

In lower vascular plants as well as in most gymnosperms the events leading to chloroplast differentiation can occur both in light and in darkness. On the contrary, in the angiosperms, light is a key factor for chloroplast biogenesis and the build-up of the thylakoid system. In these more evolved plants, light, by phytochrome mediation, induces the expression of different nuclear genes (photo-genes) coding for essential chloroplast components. Light, for instance, is required for the transcription of the *Cab* genes encoding numerous chlorophyll *a/b*-binding proteins. Moreover, chlorophyll is not produced in the dark because one of the final steps of its biosynthetic pathway, precisely the protochlorophyllide (Pchlide) to chlorophyllide (Chlide) reduction, is carried out by the enzyme Pchlide oxidoreductase (POR) which, in flowering plants, has become light dependent. As a consequence, in dark-grown (etiolated) seedlings of angiosperms, peculiar organelles, named "etioplasts," originate from proplastids in cells that would become green in the presence of light.

### Etioplasts

Etioplasts, which are the chloroplast counterparts in darkness, can be formed in nature during the first phase of plantlet growth, before the emergence from soil. An inner membrane system very different from the thylakoid develops in etioplasts (Figure 4). Most membranes have a tubular arrangement and aggregate in a tridimensional semicrystalline network of interconnected tubules, defined "prolamellar body," from which some lamellar membranes (prothylakoids) extend. The prolamellar body membranes contain the

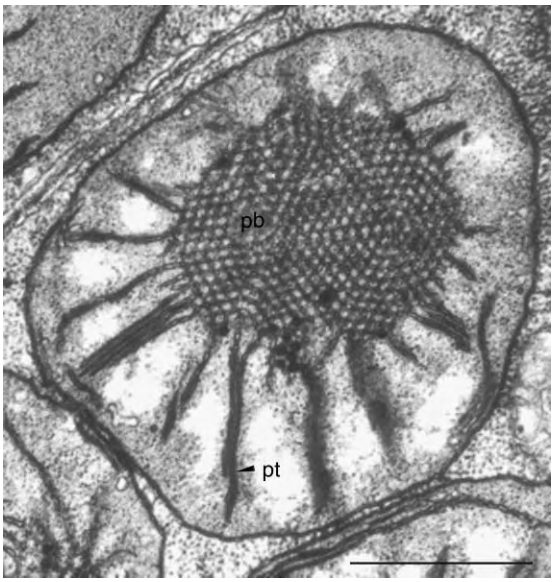



**FIGURE 4** TEM micrograph of an etioplast from a dark-grown bean cotyledon. The inner tubular membranes are arranged in a semicrystalline prolamellar body (pb) from which lamellar prothylakoids (pt) extend (bar = 1 μm).

POR associated with Pchlide and NADPH to form a stable "ternary complex," and are particularly rich in MGDG, which favors the tubular arrangement due to its cone- shaped molecular configuration.

Etioplasts exposed to light convert to chloroplasts. The first event of this greening process is the Pchlide reduction that the photo-activated POR carries out by using the NADPH of the ternary complex. Successively the etioplast membranes rearrange to form primary thylakoids and then a massive synthesis *de novo* of chlorophylls and membranes leads to the formation of the well organized and photosynthetically competent chloroplast.

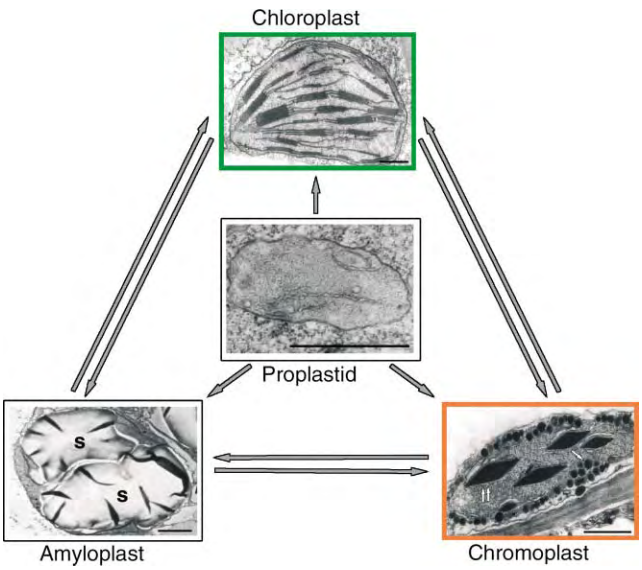



**FIGURE 5** The cyclic model of plastid interconversions. The amyloplast from a cell of *Raphanus* hypocotyl contains large starch grains (s). Globular (arrow) and crystalline (double arrow) carotenoid masses are inserted in the chromoplast from a cell of *Ranunculus* petal. Chloroplast and proplastid are from a mesophyll cell and a basal meristem cell of a maize leaf, respectively (bars = 1 μm).

## Plastids

The chloroplast belongs to the organellar class of plastids. In lower photoautotroph organisms, like algae, which do not possess cell types with very distinct functions, the photosynthetic chloroplast has remained the sole kind of plastid. On the contrary, in terrestrial plants, which evolved highly specialized tissues and organs, different kinds of plastids with specific structures and functions arose from the original chloroplast. In these plants the class of plastids, besides the green chloroplasts, includes uncolored amyloplasts, whose function is to store large amounts of starch (Figure 5) and yellow-red-brown chromoplasts which synthesize a lot of carotenoids (Figure 5) and have attractive functions. Apart from the lack of thylakoids, these non-photosynthesizing plastids share common characteristics with the chloroplasts, like the envelope membranes, the prokaryotic DNA and the 70S ribosomes. Furthermore, they maintain several biosynthetic pathways carried out by enzymes inserted in the stroma or bound to the envelope, such as those of fatty acids and terpenoids.

All the plastids can differentiate from proplastids. However, the possibility also exists that a plastid derives from the conversion of another kind of plastid, according to the "cyclic model" (Figure 5) proposed already by Schimper at the end of 1800. These reversible plastid interconversions depend on cell developmental programs as well as on endogenous (hormones and nutrients) or environmental (light and temperature) signals. Only the chromoplasts (named gerontoplasts) derived from chloroplast degeneration during a green tissue senescence cannot undergo further conversion.

## See Also the Following Articles

Chlorophylls and Carotenoids • Chloroplast Redox Poise and Signaling • Cytochrome $b_6f$ Complex

## Glossary

**binary fission** Mechanism, similar to that occurring in bacteria, by which the chloroplast divides independently from the cell division.
**envelope** Pair of concentric membranes enclosing the chloroplast.
**etioplast** Organelle which is the counterpart of chloroplast and develops in dark-grown seedlings of flowering plants.

**prolamellar body** Tridimensional semicrystalline aggregate of the etioplast tubular membranes.

**thylakoids** Flattened cisternae or saccules intercommunicating, which form the closed inner membrane system of chloroplast.

## Further Reading

Cornah, J., Terry, M. J., and Smith, A. G. (2003). Green or red: What stops the traffic in the tetrapyrrole pathway. *Trends Plant Sci.* **5**, 224–230.

Goldschmidt-Clermont, M. (1998). Coordination of nuclear and chloroplast gene expression in plant cells. *Internat. Rev. Cytol.* **177**, 115–179.

Jarvis, P. (2003). Intracellular signalling: The language of the chloroplast. *Curr. Biol.* **13**, R314–R316.

Kleinig, H. (1989). The role of plastids in isoprenoid biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 39–59.

Martin, W., and Herrmann, R. G. (1998). Gene transfer from organelles to the nucleus: How much, what happens, and why? *Plant Physiol.* **118**, 9–17.

McFadden, G. I. (1999). Endosymbiosis and evolution of the plant cell. *Curr. Opin. Plant Biol.* **2**, 513–519.

Ohlrogge, J., and Browse, J. (1995). Lipid biosynthesis. *Plant Cell* **7**, 957–970.

Sundqvist, C., and Dahlin, C. (1997). With chlorophyll pigments from prolamellar bodies to light-harvesting complexes. *Physiol. Plant.* **199**, 748–759.

Willows, R. D. (2003). Biosynthesis of chlorophylls from protoporphyrin IX. *Net. Prod. Rep.* **20**, 327–341.

## Biography

Nicoletta Rascio is a Professor of plant physiology at the Department of Biology of the University of Padova (Italy). Her principal research interest is in the biogenesis, organization, and functionality of the photosynthetic apparatus in algae and higher plants at the ultrastructural, molecular, biochemical, and physiological level, in normal and stressful environmental conditions. In this field she has published more than 100 papers in mainstream journals.

# Cholesterol Synthesis

**Peter A. Edwards**
*University of California Los Angeles, Los Angeles, California, USA*

**Cholesterol is a critical component of eukaryotic cell membranes and a precursor of steroid hormones, oxysterols, and bile acids that have important roles in cell signaling and/or lipid absorption. In addition, intermediates in the cholesterol biosynthetic pathway are diverted to the synthesis of diverse polyisoprenoids that have important functions in the cell. Thus, it is not surprising that the cholesterol biosynthetic pathway is highly regulated. Drugs, collectively called statins, that inhibit the rate-limiting enzyme of cholesterol synthesis, have been shown to both reduce plasma LDL cholesterol levels and impair the progression of atherosclerosis and coronary artery disease.**

## Properties of Cholesterol

Cholesterol is a cyclic hydrocarbon that can be esterified with a fatty acid to form a cholesteryl ester. Both cholesterol and cholesteryl esters are lipids and are essentially insoluble in aqueous solution but soluble in organic solvents. Excess cholesterol esters are stored as lipid droplets within the cytosol. Such droplets are prevalent in steroidogenic tissues where they serve as precursors of the steroid hormones. In eukaryotic cells, cholesterol is "solubilized" as a result of its interaction with either membrane phospholipids or with phospholipids and bile acid micelles in the gall bladder. In blood, cholesterol and cholesterol esters are "solubilized" within lipoprotein complexes. These lipoproteins, that include low density lipoprotein (LDL), very low density lipoprotein (VLDL), chylomicrons and high density lipoprotein (HDL), function to transport insoluble lipids around the body.

## Functions of Cholesterol

### Membrane Structure

Cholesterol is absent from prokaryotic cells. However, cholesterol plays an essential structural role in maintaining the fluidity of eukaryotic cell membranes. Cholesterol is not equally distributed in all membranes; the membranes of mitochondria, peroxisomes, and endoplasmic reticulum are cholesterol-poor, whereas the plasma membrane is enriched in the sterol. However, the concentration of cholesterol varies significantly even within the plasma membrane; it is highly enriched in two specialized areas termed lipid rafts and caveolae. Since many receptors are localized to these cholesterol- and sphingomyelin-rich domains, it has been suggested that lipid rafts and caveolae function as "signaling gateways" into the cell. The myelin sheath that surrounds nerves has the highest cholesterol concentration.

### Precursor of Signaling Molecules

As shown in Figure 1, cholesterol is a precursor of bile acids, steroid hormones, and oxysterols (oxidized cholesterol). These cholesterol metabolites function to activate specific nuclear receptors that control many metabolic and developmental processes. In addition, intermediates in the cholesterol biosynthetic pathway are themselves precursors for other critical pathways. For example, 7-dehydrocholesterol is a precursor of vitamin D, and farnesyl diphosphate is a precursor of geranylgeranyl diphosphate, ubiquinone, Heme a, and dolichols (Figure 1). Although beyond the scope of this article, it is now clear that modification (prenylation) of many proteins by either farnesyl diphosphate or geranylgeranyl diphosphate is critical for cell survival. Thus, hypolipidemic drugs (e.g., statins) that inhibit HMG-CoA reductase activity must be used at doses that result in partial, not total, inhibition of the pathway.

## Sources of Cellular Cholesterol

### Diet and Lipoproteins

Insects are cholesterol auxotrophs and must obtain all their cholesterol from the diet. In contrast, other eukaryotes obtain cholesterol from two sources; the diet and endogenous synthesis. Mammals absorb 40–50% of the cholesterol in their diet and transport it to the liver and other tissues in lipoproteins. The plasma lipoproteins bind to cell surface receptors prior to the delivery of the lipid cargo to the cell. Such receptors include the LDL receptor, SR-B1, SR-A, and CD36.

 © 2004, Elsevier Inc. All Rights Reserved. 

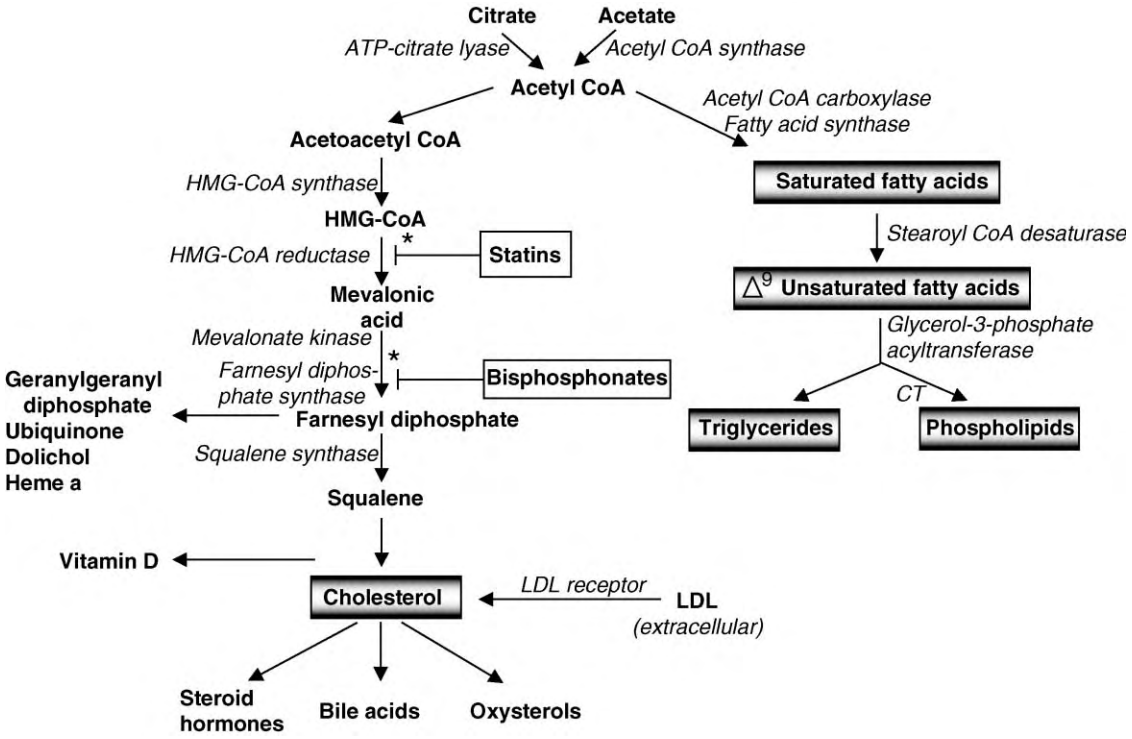



**FIGURE 1** The cholesterol and fatty acid biosynthetic pathways are regulated by SREBPs. A few of the intermediates and enzymes of the cholesterol and fatty acid synthetic pathways are shown. Genes that are preferentially regulated by SREBP-2 (left side) or SREBP-1c (right side) are italicized. The inhibition of HMG-CoA reductase or farnesyl diphosphate synthase by statins and bisphosphonates, respectively, is indicated by *, many intermediates and enzymes have been omitted for simplicity, such as CT, CTP, phosphocholine cytidyltransferase.

The LDL receptor is expressed on the surface of most cells and plays a particularly important role in cholesterol homeostasis. This transmembrane protein binds cholesterol-rich LDL and facilitates the delivery of cholesterol into the cell via specialized areas on the cell surface called clathrin-coated pits. However, virtually all mammalian cells also synthesize cholesterol (Figure 1).

## Cholesterol Synthesis

There are many reviews on the biosynthesis of cholesterol, possibly in part because work in this area has been extensive and has led to numerous Nobel prizes. All mammalian cells, with the exception of mature red blood cells, utilize more than 30 enzymes to convert the 2 carbon acetate to the 27 carbon cholesterol via isoprenoid intermediates (Figure 1). The rate-limiting enzyme in this pathway, HMG-CoA reductase, is the target for many hypolipidemic drugs (Figures 1 and 2). Together, the cholesterol biosynthetic pathway and the LDL receptor pathway provide sufficient cholesterol necessary for cell growth and division. These two pathways are coordinately controlled by an exquisite mechanism that "senses" cellular cholesterol levels. The mechanism involves the generation of three functionally active transcription factors termed sterol regulatory element binding proteins (SREBPs).

### Transcriptional Control of Cholesterol Synthesis

There are two SREBP genes, SREBP-1 and SREBP-2. The SREBP-2 gene produces one protein that preferentially activates genes involved in cholesterol homeostasis. In contrast, two proteins, SREBP-1a and SREBP-1c are produced from the SREBP-1 gene. SREBP-1c preferentially activates genes involved in fatty acid synthesis. SREBP-1a activates both pathways but is expressed at relatively low levels. Newly synthesized SREBP proteins contain two transmembrane domains that form a hairpin-like structure and anchor the proteins in the endoplasmic reticulum, outside the nucleus (Figure 2). If these proteins are anchored to membranes outside the nucleus, how can they function as transcription factors in the nucleus?

The answer came from a series of elegant studies initiated in the laboratory of Goldstein and Brown in Dallas. They showed that generation of nuclear-localized SREBPs requires additional proteins named Insig-1, Insig-2, SCAP, the site 1 protease (S1P), and the site 2 protease (S2P) (Figure 2). The roles of these proteins vary; SCAP functions both as a cholesterol sensor and as an escort protein to transport SREBP to the Golgi where two proteases cleave SREBPs to release the soluble amino terminal fragment. The process is also regulated by the two Insig proteins, which function to

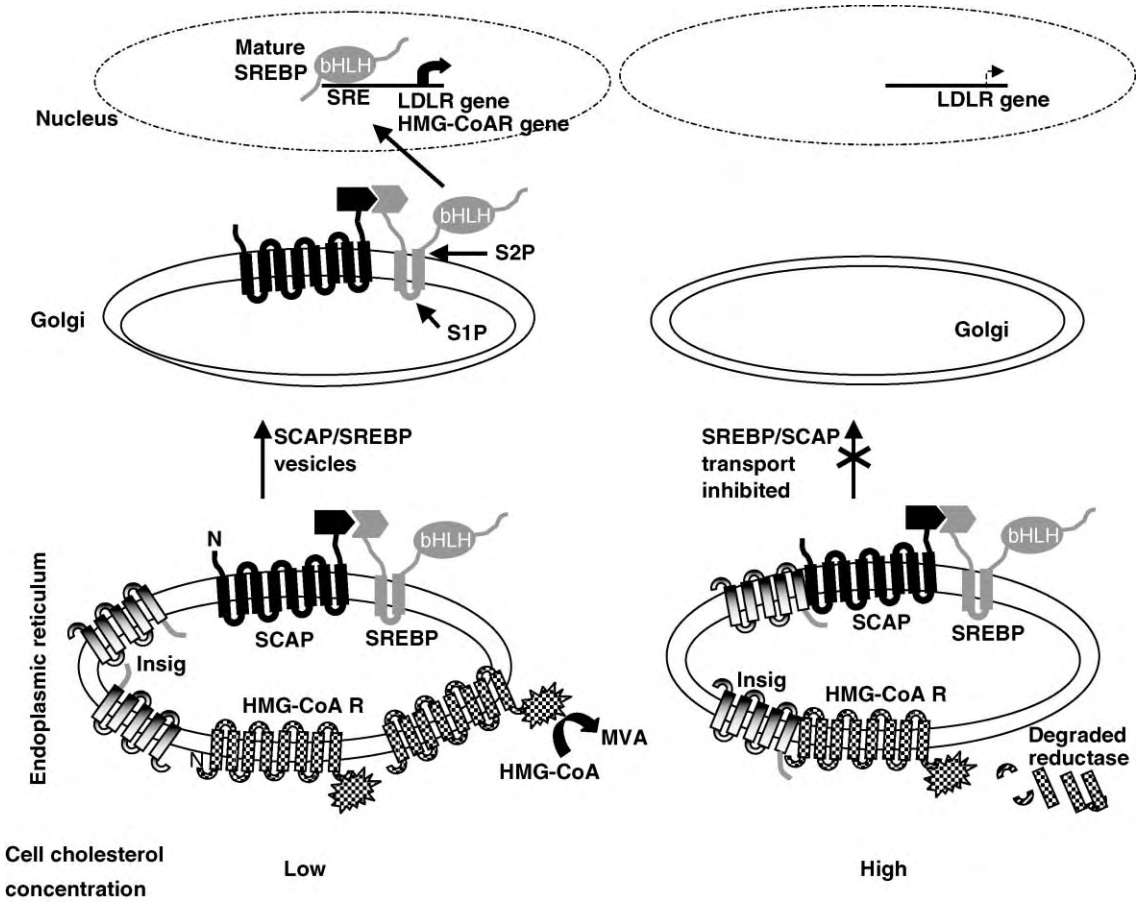



**FIGURE 2** Mechanisms involved in the nuclear targeting of SREBP-2 and the altered stability of HMG-CoA reductase. The left side represents conditions where the cholesterol concentration in the cell is low. Under these conditions, SREBP-2 is targeted first to the Golgi and then to the nucleus where it activates many SREBP-2 target genes. For simplicity, only the LDL receptor and HMG-CoA reductase (HMG-CoA R) genes are shown. HMG-CoA reductase protein also accumulates in the endoplasmic reticulum where it catalyzes the reduction of HMG-CoA to mevalonic acid (MVA). The right side represents conditions where cellular cholesterol levels are higher than normal. Under these cholesterol-enriched conditions, Insig complexes with SCAP/SREBP and inhibits the maturation and nuclear localization of SREBP-2. In addition, the rate of degradation of HMG-CoA reductase is increased. The degradation is preceded by the formation of an Insig/HMG-CoA reductase complex. Details are given in the text.

control the release of SCAP/SREBP from the endoplasmic reticulum (Figure 2). In brief, when cells become "cholesterol-poor," for example, after addition of a drug that inhibits cholesterol synthesis, a SCAP/SREBP-2 heterodimeric complex is transported in vesicles that bud from the endoplasmic reticulum and then fuse with the Golgi (Figure 2). The Golgi contains two resident proteases, S1P and S2P, that sequentially cleave the newly arrived SREBP-2 protein; first S1P cleaves SREBP into two parts by cleaving the hairpin loop in the lumen of the Golgi, then S2P cleaves the protein at a second site to release a soluble amino-terminal domain (Figure 2). This soluble amino-terminal fragment, containing a basic helix-loop-helix-leucine zipper transactivation domain (bHLH), migrates into the nucleus where it binds to specific DNA sequences termed sterol responsive elements (SREs) and activates transcription. SREs have been identified in the promoters of many genes involved in cholesterol and fatty acid and lipid synthesis. Such genes include HMG-CoA reductase, HMG-CoA synthase, farnesyl diphosphate synthase, squalene synthase, and the LDL receptor (Figure 1). In summary, a transient decrease in cellular cholesterol levels leads to increased levels of transcriptionally active SREBP-2 in the nucleus and activation of genes involved in cholesterol synthesis and uptake of LDL. The net result is a rapid return of the cellular sterol levels to normal.

In contrast, when cells accumulate cholesterol, the SCAP/SREBP heterodimer complexes with another protein called Insig. Since Insig is a resident protein of the endoplasmic reticulum, the Insig/SCAP/SREBP-2 complex remains localized to this membrane (Figure 2). Consequently, transport of SREBP-2 to the Golgi and subsequent cleavage and nuclear localization of the protein is impaired. As a result, transcription of genes encoding the LDL receptor and cholesterogenic

genes is low. Such changes in gene expression will prevent or reduce subsequent cholesterol accumulation in the cell.

Although beyond the scope of this review, SREBPs, especially SREBP-1c, also activate genes involved in the synthesis of saturated and unsaturated fatty acids, triglycerides, and phospholipids (Figure 1).

### Regulated Degradation of HMG-CoA Reductase

HMG-CoA reductase catalyses the NADPH-dependent reduction of HMG-CoA to mevalonic acid (MVA in Figure 2). It is considered to be the rate-limiting enzyme of the cholesterol biosynthetic pathway. Thus, changes in the activity of the enzyme are paralleled by changes in cholesterol synthesis. The activity of the enzyme is regulated by changes in transcription, translation (mechanism unknown) and protein stability.

HMG-CoA reductase contains two domains; an eight transmembrane spanning region that localizes the protein to the endoplasmic reticulum and a carboxy-terminal domain that projects into the cytosol and contains all the catalytic activity (Figure 2). The half-life (a measure of protein stability) of the enzyme varies at least tenfold, depending on the concentration of cholesterol and isoprenoids in the cell. When cellular cholesterol levels are low the enzyme is relatively stable (half-life $\sim$10 h). Thus, active HMG-CoA reductase enzyme accumulates in the endoplasmic reticulum in response to (1) a slow rate of degradation of the protein and (2) increased transcription and enzyme synthesis (Figure 2). Since HMG-CoA reductase is the rate-limiting enzyme, the net result is increased cholesterol synthesis.

However, when cellular cholesterol levels increase, the rate of degradation of HMG-CoA reductase protein is enhanced $\geq$10-fold (half-life $<$45 min) by a process that requires both a sterol and an unidentified non-sterol derived from farnesyl diphosphate (Figure 1). Recent studies have shown that Insig-1 forms a complex with the transmembrane domains of HMG-CoA reductase prior to the ubiquination and degradation of the enzyme in proteosomes (Figure 2).

Thus, Insig appears to have a dual role in cholesterol-enriched cells; it interacts with HMG-CoA reductase to promote degradation of the enzyme, and with SCAP to retain SREBP-2 in the endoplasmic reticulum (Figure 2). It seems likely that these interactions may depend on the partially conserved "sterol-sensing domains" identified in both HMG-CoA reductase and SCAP. In summary, cholesterol-loaded cells have low levels of HMG-CoA reductase protein (and hence low rates of cholesterol synthesis) because (1) the enzyme is rapidly degraded and (2) transcription of the gene is low as a result of the retention of SREBP-2 in the endoplasmic reticulum (Figure 2, right side). Such changes will minimize subsequent cholesterol accumulation in the cell.

## Effect of Mutations in the Cholesterol Synthetic Pathway

Mutations in the cholesterol synthetic pathway are rare because they are usually incompatible with life. Mutations in genes encoding mevalonic kinase (an early enzyme in the pathway) and 7-dehydrocholesterol (a late enzyme in the pathway) result in mevalonic acid urea and Smith–Lemli–Opitz syndrome, respectively. It should be emphasized that patients with mevalonic acid urea have residual mevalonic acid kinase activity, sufficient to provide isprenoid intermediates that are necessary for cell survival. Indeed, a complete blockage of early steps in cholesterol biosynthesis leads to embryonic death. Patients with mutations in genes that result in impaired cholesterol synthesis exhibit various neurological dysfunctions. Such observations only emphasize the importance of cholesterol to the neurological system.

## Inhibitors of Cholesterol Synthesis; Treatment for Hyperlipidemia and Osteoporosis

### Statins

Elevated LDL cholesterol levels (hypercholesterolemia) are relatively common in the West. Hypercholesterolemia is associated with the development of atherosclerosis, coronary heart disease, myocardial infarction and increased mortality. One class of drugs, referred to collectively as statins, have proven to be extremely effective in lowering plasma LDL levels and in reducing the progression of atherosclerosis and clinically associated problems.

Mevinolin is a fungal metabolite that is structurally related to HMG-CoA. It was originally discovered in 1976 by Akira Endo in Japan and shown to be a potent competitive inhibitor of HMG-CoA reductase; it binds to the enzyme with a $K_i \sim 10^{-9}$ M, compared to a $K_m$ of $\sim 10^{-6}$ M for the natural substrate HMG-CoA. Subsequent studies by many investigators led to the identification of a number of natural and synthetic compounds that also inhibit the enzymatic activity of HMG-CoA reductase. These drugs are collectively known as statins. When taken orally, these drugs are absorbed and targeted to the liver where they bind and partially inhibit HMG-CoA reductase and cholesterol synthesis. The liver cells "sense" this decrease in newly synthesized cholesterol and respond by enhancing the cleavage and nuclear localization of SREBP-2 (Figure 2). As a result, expression of a number of genes, including the LDL receptor and HMG-CoA reductase are increased. The increased expression of the LDL receptor

protein on the hepatocyte cell surface results in increased clearance of cholesterol-rich LDL from the plasma into the liver. This is the molecular basis for the clinical effects of the drug. Fortunately, the liver does not become loaded with lipid because it converts the LDL-derived cholesterol to bile acids and excretes the latter in the bile. In summary, oral administration of statins results in an increased rate of removal of LDL from the blood and a 30–50% decline in blood LDL cholesterol levels. Such a change has been shown to reduce both the progression of coronary atherosclerosis and the incidence of myocardial infarction and death.

A number of epidemiological studies have recently reported that statins also reduce osteoporosis and improve the status of patients with Alzeimers. However, at the current time, these latter conclusions are highly controversial and additional studies will be necessary to determine whether these drugs have such additional benefits.

## Bisphosphonates

Bisphosphonates are potent inhibitors of bone resorption and are used widely to treat osteoporosis. After absorption, these drugs are targeted to bone, where they are taken up by osteoclasts, the bone-resorping cells. The bisphosphonates inhibit farnesyl diphosphate synthase and isoprenoid synthesis (Figure 1). As a result, the osteoclasts undergo apoptosis and bone resorption is attenuated.

## See Also the Following Articles

Fatty Acid Oxidation • Fatty Acid Synthesis and its Regulation • LipoProteins, HDL/LDL

## Glossary

**HMG-CoA reductase** 3-Hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme of cholesterol biosynthesis.

**SCAP** SREBP cleavage activating protein. SCAP is required for the transport of SREBP from the endoplasmic reticulum to the Golgi.

**SREBP** Sterol regulatory element binding protein, a transcription factor that is proteolytically processed before entering the nucleus and binding to a *cis* element on the DNA, termed sterol regulatory element (SRE).

**statins** A class of drugs that inhibit the activity of HMG-CoA reductase.

## Further Reading

Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002). SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109**, 1125–1131.

Osborne, T. F. (2001). CREating a SCAP-less liver keeps SREBPs pinned in the ER membrane and prevents increased lipid synthesis in response to low cholesterol and high insulin. *Genes Dev.* **15**, 1873–1878.

Porter, F. D. (2002). Malformation syndromes due to inborn errors of cholesterol synthesis. *J. Clin. Invest.* **110**, 715–724.

Sever, N., Yang, T., Brown, M. S., Goldstein, J. L., and Debose-Boyd, R. A. (2003). Accelerated degradation of HMG CoA reductase mediated by binding of Insig-1 to its sterol-sensing domain. *Mol. Cell* **11**, 25–33.

Tobert, J. A. (2003). Lovastatin and beyond: The history of the HMG-CoA reductase inhibitors. *Nat. Rev.* **2**, 517–526.

## Biography

Dr. Peter Edwards is a Professor of both Biological Chemistry and Medicine at UCLA. His principal interests are centered on the roles of oxysterols and bile acids as agonists of two nuclear receptors, LXR and FXR. He holds a Ph.D. from the University of Liverpool, United Kingdom and received postdoctoral training at Stanford University. His group has purified/cloned and studied the regulation of a number of enzymes involved in cholesterol and isoprenoid biosynthesis.

# Chromatin Remodeling

**Eric Kallin and Yi Zhang**
*University of North Carolina, Chapel Hill, North Carolina, USA*

**A cell's ability to regulate gene expression has been directly linked to DNA packaging within the nucleus. The active process involved in the alteration of DNA packaging has been termed chromatin remodeling. Several types of enzyme complexes have been identified which are capable of altering the packaging state and thus affecting gene expression. To date, three distinct chromatin-remodeling mechanisms, including ATP-dependent nucleosome remodeling, covalent modification of histones, and histone variant incorporation, have been identified and each has been linked to gene expression by regulating chromatin dynamics. This article will briefly describe these three mechanisms as well as explain their relationship with gene expression.**

## Chromatin Basics

Researchers have known that certain proteins, called histones, were closely associated with DNA even before DNA was identified as the genetic material. When DNA was identified as the genetic material, it was thought that the histones only functioned as scaffolding for the wrapping of DNA so that the long stretch of DNA (3.2 cm long for human) can be contained within the cell nucleus (with a diameter of $4 \times 10^{-3}$ cm). The first breakthrough in the studies of the role of histones in organizing DNA was made by Roger Kornberg in 1974. He found that core histones exist as defined octamers that are associated with DNA at nearly even intervals similar to that of "beads on a string." The basic repeating unit of chromatin, called the nucleosome, includes two copies of each of the four core histones, H2A, H2B, H3, and H4. Further biochemical analysis has shown that each histone octamer is encircled almost twice by DNA of 146 bp. Large numbers of noncovalent bonds form between the DNA and the histone proteins resulting in a stable complex. A fifth histone protein, called H1, binds to the linker DNA of two adjacent nucleosomes to maintain the higher-order chromatin structure.

The function of a given cell is largely determined by the sets of genes that it expresses. Cells control their gene expression mainly at the transcriptional level which involves binding of specific transcription factors to DNA elements located at the promoter regions of a given gene. Compacting of DNA into chromatin impedes access to transcription factors thus stopping gene expression. Therefore, regulation of chromatin structure and nucleosome placement along the DNA strand has a significant impact on gene expression. Studies in the past 10 years have revealed at least two groups of enzymes capable of modulating chromatin structure. The first group involves factors utilizing the energy derived from ATP hydrolysis to "remodel or relocate nucleosomes." The second group involves enzymes that covalently modify core histone proteins. Recent studies also indicate that substitution of regular histones with their variants also affects chromatin structure.

## ATP-Dependent Nucleosome Remodeling

ATP-dependent nucleosome remodeling refers to the use of energy derived from ATP hydrolysis to fuel a reaction that results in the translocation of a nucleosome to another portion of the DNA molecule. If a nucleosome is positioned in such a way that an important regulatory DNA element is blocked from transcription factor binding, relocating that nucleosome to another portion of DNA could expose the binding site and result in gene expression. ATP-dependent nucleosome-remodeling factors usually exist in multiprotein complexes. A common feature of these protein complexes is that they all contain a subunit capable of hydrolyzing ATP. Based on the ATPase subunit, the known remodeling factors have been grouped into three families.

### Remodeling Enzymes

#### SWI/SNF Family

The SWI/SNF family of proteins was first identified in budding yeast by genetic screens to identify genes that are involved in mating type switch (SWI) and sucrose nonfermenting (SNF). Sequence analysis revealed that *SNF2* and *SWI2* encode the same protein which is

 © 2004, Elsevier Inc. All Rights Reserved. 

the founding member of an ATPase family. Biochemical and genetic studies led to the identification of an 11-subunit complex, named the SWI/SNF complex, capable of increasing the accessibility of nucleosomal DNA by transcription factors. Components of this complex have been linked to ATPase activity (Snf2), nonspecific-DNA-binding activity (Swi1), and nuclear-structure binding (Arp7 and Arp9). A highly related complex called remodels structure of chromatin (RSC) has also been identified in budding yeast. The ATPase subunit of RSC has been named Sth1 and is homologous to Snf2 of the SWI/SNF complex. In comparison with the SWI/SNF complex, RSC is a much more abundant complex. Genes that encode components of the RSC complex are essential for yeast survival. Protein complexes homologous to the yeast SWI/SNF complex have been identified in other multicellular organisms such as *Drosophila* and human.

### ISWI Family

Using a functional assay that measures activities capable of disrupting regular nucleosome spacing, Carl Wu and colleagues purified the nucleosome remodeling factor (NURF) complex from *Drosophila* embryo extracts. The complex is composed of four subunits, including the ATPase, imitation switch (ISW) I. In addition to NURF, ISWI was also found to be present in two other *Drosophila* remodeling factors, ACF (ATP-utilizing chromatin assembly and remodeling factor) and CHRAC (chromatin accessibility complex). Although ISWI by itself is able to remodel nucleosomes *in vitro*, it functions in one of the three complexes *in vivo*. At least three homologous complexes containing the ISWI homologue hSNF2h have been identified in humans and two complexes containing the ATPase ISW1 and ISW2 have been identified in budding yeast.

### Mi-2 Family

Mi-2 was initially identified as a nuclear autoantigen in patients with dermatomyositis. Two forms, Mi-2$\alpha$ (or CHD3) and Mi-2$\beta$ (or CHD4), which are 72% identical at the amino acid level, exist in human cells. They both belong to the chromo-helicase-DNA-binding (CHD) protein family, which includes at least four human family members. In addition to the conserved SWI2/SNF2 helicase/ATPase domain present in the ATPase subunit of other nucleosome remodeling factors, the Mi-2 proteins also include two plant homeodomain (PHD) zinc-fingers, two chromo domains, and an HMG-like domain. Biochemical studies indicate that Mi-2 proteins exist in a protein complex named NuRD that possesses both nucleosome remodeling and histone deacetylase activities. Both HDAC1 and HDAC2 (histone deacetylase 1 and 2) are components of the NuRD complex. They interact with the PHD fingers of the Mi-2 protein. Recent studies indicate that the NuRD complex can associate with the methyl-CpG-binding protein MBD2 to form the MeCP1 complex. Thus, the NuRD complex links nucleosome remodeling and histone deacetylation to DNA methylation-mediated gene silencing. Although NuRD-specific subunits were not found in the budding yeast, homologues of Mi-2 and MTA2 proteins exist in *Drosophila* and *Caenorhabditis elegans*. Therefore, it is likely that the functional counterpart of NuRD was evolutionarily conserved in multicellular organisms.

## Remodeling Mechanisms

Although all of the remodeling complexes identified so far have a component that possesses ATPase activity, the properties of their respective ATPases appear different. While the SWI2/SNF2 protein and SWI/SNF complexes exhibit DNA-stimulated ATPase activity, the ATPase activity of ISWI alone or in complex is only stimulated by nucleosomes. Moreover, SWI/SNF complexes seem to induce much more dramatic structural changes in nucleosomes than the ISWI complexes. These differences suggest that different remodeling factors may use different remodeling mechanisms. Based on the observation that in the presence of SWI/SNF and ATP, mononucleosomes can generate a novel nucleosome dimer species which has loosened histone–DNA contacts, an interconversion model was proposed to explain how the human SWI/SNF complex works. It is believed that interconversion between the two nucleosome states occurs constantly (Figure 1A). The SWI/SNF complex simply lowers the energy required for this interconversion. An alternative "octamer transfer" model was proposed to explain the mechanism of the RSC function (Figure 1B). The basis for this model was the observation that RSC can completely disrupt histone–DNA contact and transfer the histone octamer to another molecule of DNA. As explained above, histones associate with DNA through a collection of noncovalent interactions. Given the large energy requirement for breaking the histone–DNA interactions, the "octamer transfer" model appears unlikely. Therefore, a third "octamer sliding" model (Figure 1C) was proposed for NURF and CHRAC. In contrast to RSC, NURF and CHRAC increase the mobility of histone octamers along DNA without affecting the integrity of the octamer. The basis of this model is that both NURF and CHRAC can promote nucleosome redistribution to one favored position. Whether any of the models are correct *in vivo* remains to be determined.

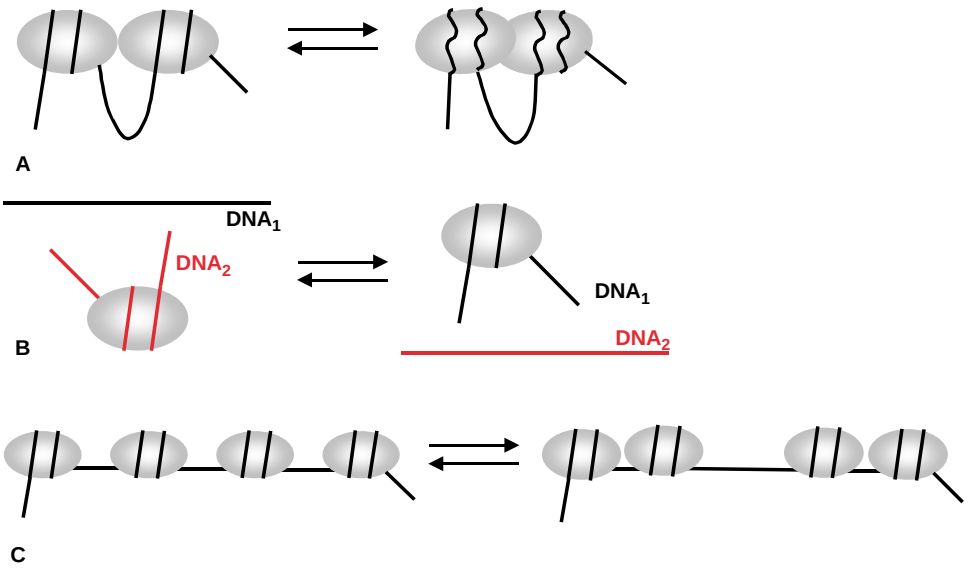



**FIGURE 1** Schematic representation of the proposed ATP-dependent remodeling mechanisms. (A) Interconversion model (nucleosome destabilization model). (B) Octamer transfer model showing a histone octamer being transferred from one molecule of DNA to another. (C) Octamer sliding model showing the regularly spaced nucleosomes are disrupted. All three models would result in segments of DNA becoming more accessible to additional protein complexes.

### Targeting of Activity

The above-described remodeling factors were first thought to bind and randomly scan DNA for nucleosome targets. However, this view changed when the BRG1 remodeling complex was found to interact with the glucocorticoid receptor, a sequence-specific DNA-binding transcription factor. Recruitment of a remodeling factor by a sequence-specific transcription factor could result in localized nucleosome remodeling and transcriptional activation. Similarly, the methyl-CpG-binding protein MBD2 could recruit the NuRD complex to patches of methylated DNA resulting in targeted remodeling, histone deacetylation, and consequently repression of methylated promoters. A third targeting strategy has developed in SWI/SNF through its ARP7/9 subunits. These proteins have distinct domains that can bind to actin and actin-related proteins which in turn target the protein complex to specific chromatin domains. A common feature of these strategies is to bring the remodeling complex into close proximity with DNA promoter sequences to achieve precise control of gene expression.

## Covalent Modifications

As an important component of the nucleosome, each core histone is composed of a structured, three-helix domain called the histone fold and two unstructured tails. Although the histone tails are dispensable for the formation of the nucleosome, they are required for nucleosome–nucleosome interaction. The core histone tails are susceptible to a variety of covalent modifications, including acetylation, phosphorylation, methylation, and ubiquitination (Figure 2). Although these modifications were identified in the early 1960s, their function was not understood until very recently. The identification of the first nuclear histone acetyltransferase (HAT) by David Allis and colleagues has led to intensive studies on the function of these histone modifications. As a result, enzymes responsible for each of the modifications have been identified and the functions of these modifications, particularly in transcriptional regulation, are beginning to be dissected.

### Acetylation

Since the discovery of histone acetylation by Allfrey and colleagues in 1964, many studies on this modification have been performed. As a result, this modification is the best-studied histone modification that has a direct effect on gene regulation. In contrast to the N-terminal $\alpha$-acetylation on many proteins, histone acetylation occurs on the $\varepsilon$-amino group of specific lysine residues in the N-terminal tails. Acetylation involves the transfer of an acetyl group from acetyl-CoA onto the $\varepsilon$-amino group of lysine residues, which neutralizes the positive charge of the histone tails and decreases their affinity for DNA. Deacetylation, on the other hand, involves the removal of the acetyl group, thus introducing a positive charge on the histone tails. Enzymes that catalyze the acetylation process are referred to as histone acetyltransferases (HATs). Enzymes that

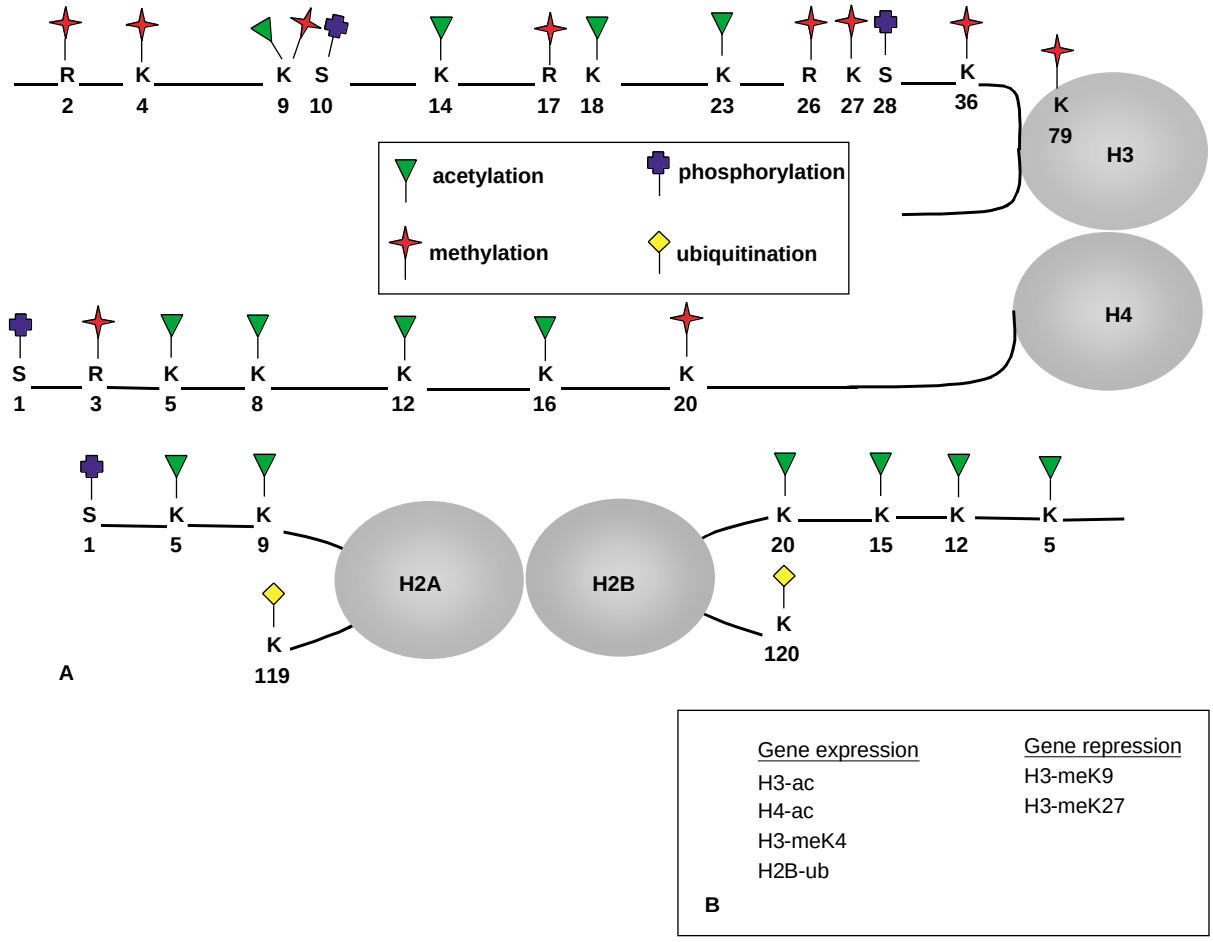



**FIGURE 2** (A) Sites of known core histone post-translational modifications. (B) List of general gene expression patterns relating to histone modifications.

catalyze the reverse process are called histone deacetylases (HDACs).

Numerous HATs and HDACs have been identified and several of these enzymes are conserved across the eukaryotic spectra. While a number of the HATs, such as Gcn5, p300, and PCAF, function as transcriptional coactivators; several HDACs function as transcriptional corepressor. In general, histone acetylation leads to the relaxation of chromatin structure and thus correlates with gene activation. In contrast, histone deacetylation leads to condensation of chromatin structure and thus correlates with gene repression. Therefore, histones in the actively transcribed chromatin region, the euchromatin, are hyperacetylated while histones in the silenced chromatin region, the heterochromatin, are hypoacetylated. It has been shown that the enzymatic activities of both HATs and HDACs are important in transcriptional regulation, as inactivation of the enzymatic activity by mutagenesis of the catalytic site impairs both the enzymatic activity and their transcriptional activity. It is believed that histone acetylation levels affect higher-order chromatin structure. Therefore, acetylation likely impacts transcription by affecting the accessibility of transcription factors to their cognate DNA.

## Methylation

First described in 1964, histones have long been known to be a substrate for methylation. Early studies using metabolic labeling followed by sequencing of bulk histones have shown that several lysine residues, including lysines 4, 9, 27, 36, and 79 of H3 and lysine 20 of H4, are the preferred sites of methylation. Recent studies indicate that histone methylation can also occur on arginine residues, including arginine 17 of H3 and arginine 3 of H4 (Figure 2). Arginine can be either mono- or dimethylated, with the latter in symmetric or asymmetric configurations. Proteins that catalyze histone arginine methylation belong to the protein arginine methyltransferases (PRMT) family. PRMT1 and PRMT4 catalyze H4-R3 and H3-R17 methylation, respectively. Accumulating evidence indicates that histone arginine methylation plays an important role in nuclear hormone-mediated transcriptional activation.

Although histone arginine methyltransferases were discovered before histone lysine methyltransferases, the latter have recently attracted more attention. Since the discovery of the first lysine methyltransferase SUV39H1, many histone methyltransferases (HMTases) that target to different lysine residues on histone H3 and histone H4 have been discovered. A common feature of these histone lysine methyltransferases is that they all contain an evolutionarily conserved SET domain, a motif shared in the *Drosophila* PEV (*p*osition *e*ffect *v*ariegation) suppressor Su(var)3-9, the *Polycomb*-group protein E(z), and the *trithorax*-group protein Trx. Recent studies, however, have uncovered that lysine 79 of histone H3, located in the globular domain, is also methylated in diverse species from yeast to human. Surprisingly, the responsible enzymes, DOT1 and its homologues, do not contain a SET domain. Thus, lysine methylation can be catalyzed by proteins with or without a SET domain.

Unlike histone acetylation which usually results in gene activation, the effect of histone methylation on transcription is site dependent. While H3-K4 methylation correlates with gene activation, methylation on H3-K9 and H3-K27 usually causes gene silencing. Recent studies indicate that even on the same site, the outcome of methylation may be different depending on whether it is a mono-, di-, or trimethylation. These site-specific or methylation state-specific effects may reflect the fact that different modifications are recognized and interpreted by different protein modules, such as the chromadomain and bromodomain, as suggested by the "histone code" hypothesis. Studies in the past three years have revealed that this modification has significant effects on multiple biological processes ranging from heterochromatin formation and transcriptional regulation to genomic imprinting, X-chromosome inactivation, and DNA methylation. How this modification results in such a diverse effect is currently unknown.

## Phosphorylation

Both the linker histone H1 and core histones undergo phosphorylation on specific serine and threonine residues (Figure 2). Phosphorylation of histones is cell-cycle dependent with the highest level of phosphorylation occurring in M phase. H1 phosphorylation occurs on both N-terminal and C-terminal domains and is linked to transcription. For example, inactivation of the MMTV promoter correlates with H1 dephosphorylation while reactivation of the promoter correlates with H1 rephosphorylation.

Recent studies indicate that phosphorylation of serines 10 and 28 of H3 are tightly regulated and are implicated in the establishment of transcriptional competence of immediate early-response genes. For example, induction of early-response genes, such as c-*fos* and c-*jun*, is concurrent with H3 phosphorylation. Both the MAP (mitogen-activated protein kinase) pathway and the stress-activated p38 pathway are involved in this process. At least two kinases, the ERKs-activated Rsk-2 kinase and the related Msk-1 (MAP- and stress-activated kinase 1), are responsible for the rapid H3 phosphorylation. Although phosphorylation of histones correlates with transcriptional activation of a small set of immediate-early genes that are rapidly turned on and off in response to extracellular signals, the bulk of histone phosphorylation occurs in M phase when genes are silenced. Recent studies indicate that mitotic histone phosphorylation correlates with chromosome condensation and is mediated by the Ipl1 and Aurora kinases.

In addition to transcription regulation, histone phosphorylation is also linked to other cellular processes such as DNA damage and apoptosis. For example, H2A.X, an H2A variant in mammalian cells, has been shown to be rapidly phosphorylated on S139 upon exposure to ionizing radiation. Interestingly, the phosphorylated form of H2A.X is localized to mega-base regions of DNA that flank sites of double-strand breaks, suggesting that this modification may function to facilitate access of repair machinery to damaged DNA. Recent studies on H2B indicate that H2B-S14 phosphorylation is tightly associated with apoptotic chromatin and that the caspase-cleaved Mst1 (mammalian sterile twenty) kinase is responsible for this modification.

## Ubiquitination

In addition to the modifications discussed above, histones and their variants can also be reversibly ubiquitinated. Unlike other modifications which result in the addition of relatively small moieties, ubiquitin is a highly conserved 76 amino acid protein. Ubiquitination occurs on the $\varepsilon$-amino group of specific lysine residues (H2A-K119 and H2B-K120). In higher eukaryotic cells, about 10% of H2A and 2% of H2B are ubiquitinated. While mono-ubiquitination is the major form of H2B ubiquitination, polyubiquitination, where ubiquitin molecules join to each other by isopeptide bonds, has been reported for H2A.

H2B ubiquitination has long been linked to transcriptionally active genes. It is possible that actively transcribing genes have an open chromatin structure exposing the C terminus of H2B to the enzymes catalyzing the addition of ubiquitin. Studies in the budding yeast have identified Rad6 as an H2B ubiquitination enzyme. Recent studies also indicate that methylation on H3-K4 and H3-K79 in budding yeast is dependent on Rad6 and H2B ubiquitination suggesting that different modifications have "crosstalk." It is believed that ubiquitination may serve as a signal for

the recruitment of other histone-modifying enzymes, such as histone methyltransferases. However, whether this is the case awaits demonstration.

## Modification Crosstalk and the "Histone Code" Hypothesis

In addition to the crosstalk between histone methylation and ubiquitination mentioned above, crosstalk also has been shown among other modifications. For example, H3-K9 methylation and H3-S10 phosphorylation inhibit each other. H3-S10 phosphorylation, on the other hand, appears to act synergistically with H3-K14 acetylation. Moreover, it has been demonstrated that methylation on H3-K9 by SUV39H1 inhibits subsequent histone acetylation by p300, while methylation on H3-K4 by SET7 facilitates subsequent histone methylation by p300. Given that histone acetylation is linked to gene expression, the differential effects of histone methylation on subsequent acetylation provide an explanation as to why methylation on different lysine residues results in different outcomes on gene expression.

Based on the observed cross-talks, David Allis and Bryan Turner have proposed a "histone code" hypothesis. This hypothesis predicts that a pre-existing modification affects subsequent modifications on histone tails and that these modifications serve as markers for the recruitment of different proteins or protein complexes to regulate diverse chromatin functions, such as gene expression, DNA replication, and chromosome segregation. The best available evidence supporting this hypothesis is derived from the identification of protein modules that recognize specific modifications on histones. For example, the bromodomain, a motif present in many chromatin-related proteins, can specifically bind to acetylated

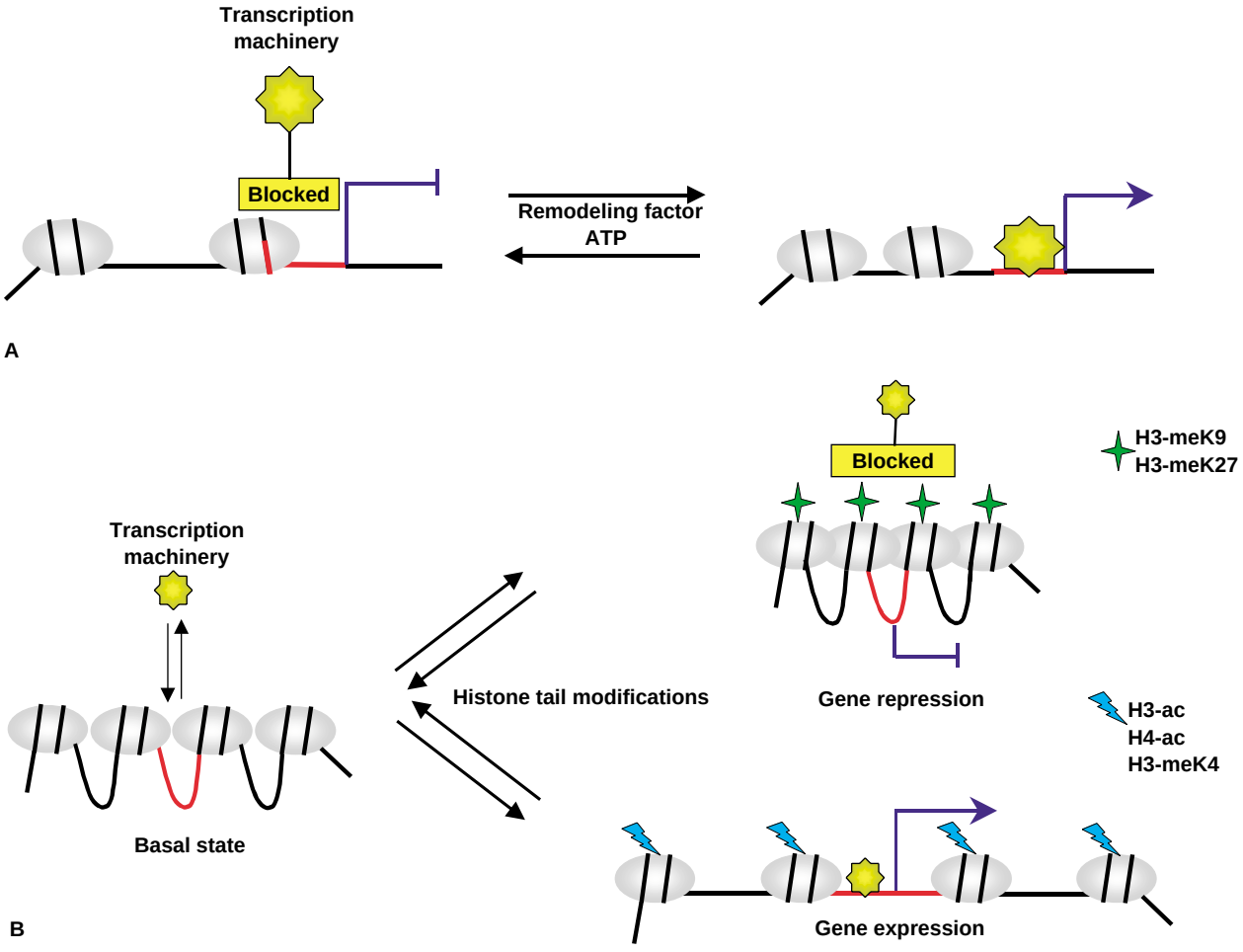



**FIGURE 3** General mechanisms of chromatin remodeling and histone modification in relation to gene expression. Sunburst represents transcriptional machinery and associated factors, red lines represent gene promoter regions, stars and lightning bolts represent different histone tail modifications. (A) ATP-dependent chromatin remodeling can lead to blocked promoters becoming accessible to transcription stimulating complexes. (B) Histone tail modifications can lead to gene repression or expression depending on the modification.

lysine residues. Similarly, chromodomain, a motif also present in many chromatin-related proteins, can specifically bind to methylated lysine residues. It is believed that the unique set of covalent modifications present on a given nucleosome serves as a "landing pad" for the recruitment of additional proteins that "read" or "interpret" the "histone code" to decide the outcome. Defining the "histone code" and identifying protein modules that read the code remain a challenge for the people in the chromatin and transcription field.

## Histone Variants

A discussion of chromatin and gene expression would not be complete without mention of histone variants. Several variations of "conventional" histone proteins have been identified in all eukaryotes from yeast to man. These variations on the histone proteins are usually found complexed in the octamer with the "normal" versions of the other histone proteins within defined regions of DNA. A well-documented H3 variant called CENP-A has been found to mark centromeres in all eukaryotic organisms studied thus far. Centromeric chromosomal regions have very few genes and are compacted into repressed heterochromatin. In contrast, the H2A variant called H2A.Z has been found to associate with inducible promoters. These are thought to play a role in the signal transduction pathways necessary for the expression of inducible genes. In addition, another H2A variant, H2A.X has been found to be specifically enriched in the proximity of double-strand break.

## Putting it All Together

While compacting DNA into a manageable structure is important for cell integrity, it also creates the problem of restricting the access of important regulators from DNA elements. To circumvent this potentially dangerous problem, the cell has developed a rather complex scaffold of histone proteins that can be altered and modified to expose important regions of the DNA. Covalent modifications of histone tails can serve to disrupt higher-order chromatin structure returning DNA to a "beads on a string" state. ATP-dependent nucleosome remodeling machinery can reveal DNA sequences masked by nucleosome interaction to important regulatory proteins, and variant histones can serve to mark complete functional domains within the chromosome (such as repressed centromeric regions) as active or inactive transcriptional regions. Studies in the past 10 years have changed our view of chromatin from a static structure to a dynamic regulatory machine that impacts all aspects of biological processes involving DNA (Figure 3). A combination of all the regulatory systems that a cell uses to modulate chromatin structure is the basis on which the complexity and diversity of life is built.

## See Also the Following Articles

Actin-Related Proteins • Centromeres • Chromatin: Physical Organization • Chromosome Organization and Structure, Overview • DNA Replication: Eukaryotic Origins and the Origin Recognition Complex • Metaphase Chromosome • Nuclear Organization, Chromatin Structure, and Gene Silencing • Ubiquitin System

## Glossary

**bromodomain** A distinct protein domain that is found in chromatin-related proteins in a variety of organisms from mammals to yeast. Bromodomain binds specifically to acetylated lysine residues on histone proteins.

**chromatin** Name given to the DNA–protein complex present in the nucleus of eukaryotic cells.

**chromodomain** The chromatin-organization-modifier domain, a conserved region of ~60 amino acids, originally identified in *Drosophila* modifiers of variegation. Recent studies indicate that this domain binds specifically to methylated lysine residues on histone proteins.

**nucleosome** The repeating unit of chromatin composed of DNA wrapped around a histone octamer consisted of two copies each of H2A, H2B, H3, and H4.

## Further Reading

Becker, P. B., and Horz, W. (2002). ATP-dependent nucleosome remodeling. *Annu. Rev. Biochem.* **71**, 247–273.

Cheung, P., Allis, C. D., and Sassone-Corsi, P. (2000). Signaling to chromatin through histone modifications. *Cell* **103**, 263–271.

Kornberg, R. D., and Lorch, Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryotic chromosome. *Cell* **98**, 285–294.

Moore, S. C., Jason, L., and Ausio, J. (2002). Elusive structural role of ubiquitinated histones. *Biochem. Cell Biol.* **80**, 311–319.

Roth, S. Y., Denu, J. M., and Allis, C. D. (2001). Histone acetyltransferases. *Annu. Rev. Biochem.* **70**, 81–120.

Strahl, B. D., and Allis, C. D. (2000). The language of covalent histone modifications. *Nature* **403**, 41–45.

Zhang, Y., and Reinberg, D. (2001). Transcription regulation by histone methylation: Interplay between different covalent modifications of the core histone tails. *Genes Dev.* **15**, 2343–2360.

## Biography

Eric M. Kallin is a graduate student in the curriculum in Genetics and Molecular Biology at the University of North Carolina at Chapel Hill. He received his B.S. in Biochemistry at the State University of New York at Geneseo. His current research, understanding the role of epigenetic modifications in cell lineage commitment, is being carried out in the laboratory of Dr. Yi Zhang.

Yi Zhang is an assistant Professor of the Department of Biochemistry and Biophysics and a member of the Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill. His research interest centers on the role of chromatin remodeling and histone tail modification in gene expression and cell lineage commitment. He obtained his Ph.D. from Florida State University in 1995. He made seminal discoveries that link nucleosome remodeling to histone tail modification. His laboratory at UNC has been responsible for the discovery of a number of novel histone methyltransferases.

# Chromatin: Physical Organization

**Christopher L. Woodcock**
*University of Massachusetts, Amherst, Massachusetts, USA*

**The term chromatin was coined by cytologists to designate the brightly staining portions of nuclei exposed to basic dyes. Although this terminology is still in effect, in the context of biological chemistry, chromatin usually refers to the complex of DNA and protein that is present in the interphase nucleus, and can be isolated for study *in vitro*. As with all biological systems, the physical organization of chromatin results from the intrinsic biochemical properties of the components as influenced by the composition of the aqueous medium. Since chromatin organization plays a critical role in many aspects of gene regulation, there is much interest in understanding the basic principles involved. Here, the current status of this challenging task is discussed in terms of the major components of chromatin, their interactions, and the complex, dynamic "biopolymer" that results.**

A crude analysis of isolated chromatin reveals a 1:1 ratio of DNA and histones, small, highly conserved basic proteins. Four species of histone (H2A, H2B, H3, and H4) contribute two copies each to the histone core of the nucleosome, a disk-shaped structure 11 nm in diameter and 6 nm in height, while histone H1 binds to the exterior of the nucleosome. Other proteins bound to DNA in chromatin are collectively known as "non-histone chromatin proteins" (NHCPs), and include both structural and enzymatic components. Thus, at the simplest level, the physical organization of chromatin consists of very long linear arrays (one for each chromosome) of nucleosomes arranged in a beads-on-a-string conformation, together with bound NHCPs. This "primary structure" is variously folded and compacted, and (in the interphase nucleus) occupies a distinct volume or "territory" with little or no intermixing with other chromosome territories. Prior to cell division, a complex series of events transforms interphase chromatin into the familiar linear metaphase chromosomes. It is clear that the arrays of nucleosomes are exposed to a wide range of local and global factors that influence the degree of chromatin compaction, and the challenge has been to understand the impact of these factors on the physical organization. There is strong evidence that features of chromatin organization such as compaction level are related to mechanisms of differential gene expression. Further, some aspects of chromatin organization are important in epigenetic inheritance, whereby properties such as differential gene silencing or activation are inherited not as differences in DNA sequence, but as specific chromatin structures and their components.

Global factors that influence the physical organization of chromatin include the ionic milieu of the nucleus, especially the concentrations of monovalent and divalent cations, and polycations such as polyamines. The combination of charge shielding and charge neutralization that results from exposure of chromatin to cations *in vitro* results in stronger intra-chromatin interactions (as the self-repulsive properties of DNA are negated) and greater compaction. Indeed, at the concentration of cations thought to be present in the living nucleus, chromatin *in vitro* tends to aggregate and essentially precipitate. Modulating the effects of cations are local variations in nucleosomes (histone variants, histone modifications), DNA modifications (chiefly cytosine methylation), and bound NHCPs (often related to histone modifications). Thus, the chromatin template has the potential to assume a continuum of compaction levels, and it is this context that its physical organization has to be viewed. In this article, the principal variables that can influence the structure and function of a given portion of the genome are outlined, after which the question of physical organization is discussed.

## Variables that may Influence Chromatin Physical Organization

### Posttranslational Modifications of Core Histones

The presence of a large variety of core histone modifications has been known for many years, but its role in chromatin was very poorly understood. Recently, however, evidence has grown that the pattern of histone modifications on an individual nucleosome or array of nucleosomes encodes information that is essential in determining a variety of functional states. Many modifications occur on the N termini of the core histones which project from the nucleosome and are

 © 2004, Elsevier Inc. All Rights Reserved. 

unstructured in the sense that they are not detected in nucleosome crystals by X-ray analysis. Some, such as acetylation and methylation of lysine residues result in the net loss of a positive charge, and may in itself result in a change in chromatin conformation. However, it is clear that the functional implications of histone modification result from far more complex effects than alterations in charge balance.

For example, H4 acetylation can occur at one or more of four lysines in the N terminus, and these modifications alone, and in combination, carry important functional signals. The net acetylation pattern is the result of the balance between histone acetylase (HAT) enzymes and deacetylase (HDAC) enzymes. Both HATs and HDACs tend to occur in large complexes that may contain proteins that serve to target the enzymatic activity to the appropriate gene(s). Specific acetylation patterns also occur on newly synthesized chromatin, and are erased after deposition as nucleosomes close to the replication fork.

Another key set of histone modifications involves the methylation of lysines on histone H3. Here, another level of complexity arises because the methyl transferases may deposit one, two, or three methyl groups, and the number of methyl groups provides another signal. Certain methylation patterns are important in providing binding sites for a group of NHCPs known as heterochromatin protein 1 (HP1). HP1 tends to accumulate on transcriptionally silent chromatin and appears to be involved in the down-regulation of genes.

## Variants of Core Histones

Typically, higher organisms have several genes for each of the core histones, some of which may be identical in sequence, while others vary in amino acid sequence. The majority of core histone genes are transcribed only during DNA replication, and are incorporated into nucleosomes in the wake of the replication fork. Others are synthesized constitutively, and incorporated into nucleosomes only at the relatively rare event of displacement of a complete histone octamer. An interesting example is provided by the replication coupled H3 and the H3.3 variant which can be deposited in both replication-coupled and replication-independent pathways, and which differ at only four amino acid positions. Single amino acid changes of H3 toward H3.3 allow replication independent deposition. H3.3 accumulates modifications that favor transcription, while H3 accumulates lysine methylations, a mark of "inactive chromatin." Although H3 is by far the most abundant variant in higher organisms, it is likely to have evolved from H3.3, which is the only H3 isoform in yeast. Another H3 variant which is highly conserved evolutionarily occurs only in centromeric chromatin.

## Histone H1

Histone H1, also known as "linker histone," plays a dominant role in establishing the compaction state of an array of nucleosomes, as well as influencing the conformation. H1 has three domains: a central globular domain that binds near the entry/exit site of linker DNA on the nucleosome, and extended N and C termini. The C terminus is particularly rich in the basic amino acids lysine and arginine, and thus has a strong propensity to bind DNA. H1 binding protects an extra ~20 bp of nucleosomal DNA from nuclease digestion, a protection conferred by the globular region alone. There is a general correlation between the amount of H1 in chromatin, and its ability to be transcribed, and most models of transcriptional regulation call for the displacement of H1. The silencing effect of H1 could be due to the "sealing" of two turns of nucleosomal DNA, thus preventing the unwrapping needed for RNA polymerase, or to the H1-induced physical compaction of arrays of nucleosomes or most likely to a combination of both.

Like the core histones, there are several variants of H1 in most higher organisms, but little is known about their individual properties. Genetic manipulations of mice that knock out one or two variants produce no observable phenotype, and the remaining variants are up-regulated to compensate for the loss. The loss of additional variants leads to a depletion in total H1 and eventually embryonic lethality. However, in H1-depleted nuclei, there is no general derepression of transcription, as predicted by the simple model that H1 loss should create "open" and permissive chromatin.

## NHCPs

A two-dimensional gel of the proteins bound to the chromatin of an active nucleus reveals hundreds of spots, and it is clear that there are many other proteins present in low abundance that are revealed only after staining with specific antibodies. To date, relatively few of these NHCPs have been studied in detail, and most of these are either abundant in the nucleus, or came to researchers' attention through functional studies of specific genes or classes of genes. Most NHCPs can be eluted from chromatin by treatment with 0.3–0.4 M monovalent ions (usually NaCl), in contrast to H1 which requires 0.5–0.6 M NaCl and the core histones which elute between 0.8 and 2.0 M salt. There is some evidence that the few proteins that remain bound in 2.0 M salt contribute to a nuclear skeleton (karyoskeleton, nuclear matrix), which provides structural integrity to the nucleus, and has an important dynamic role in its spatial organization as well as replication and transcription. However, despite much effort, it has not been possible to reveal an *in vivo* karyoskeleton analogous to the cytoskeleton, and the possibility that

the residual proteins in salt-extracted (and usually DNase-treated) nuclei represent an aggregation phenomenon has not been excluded. The finding that the most abundant nuclear matrix proteins are associated with the peripheral nuclear lamina or are part of the RNA transcript processing machinery has reinforced these concerns.

The ubiquitous and abundant (typically 1 per 10–15 nucleosomes) high mobility group (HMG) proteins elute from nuclei in 0.3–0.4 M salt, and were originally grouped together on the basis of their solubility properties and rapid mobility in gels. A new nomenclature based on the presence of conserved domains or "boxes" distinguishes three classes. Members of the HMG-B group share the "HMG-box" functional domain, which contains three $\alpha$-helices and, when bound to double-stranded DNA, induces a bend of $\sim 90°$. In contrast, the HMG-N family of proteins bind to nucleosomes via a nucleosome binding domain (NBD), resulting in a change of conformation, and the stimulation of transcription and replication. Finally, the HMG-A family members contain three "A–T hook" motifs that bind preferentially to the minor groove of DNA rich in AT sequences. HMG-A proteins appear to participate in a wide range of chromatin-based processes and are often up-regulated in cancerous cells. The lack of sequence specificity of all the HMG proteins suggests that they are targeted to the appropriate sites by interactions with other nuclear proteins, and evidence is accumulating for many such interactions.

The HP1 family of NHCPs, known to play a role in epigenetic inheritance, is also conserved across a wide range of eukaryotes, with three isoforms, $\alpha$, $\beta$, and $\gamma$ found in mammalian nuclei. HP1 (as well as several other important NHCPs) molecules share a "chromo" domain which binds not to DNA but to the methylated form of lysine 9 on histone H3. HP1 also interacts with numerous other proteins, including an enzyme that methylates H3. This illustrates the complex interactions and feedback loops that are probably widespread in the nucleus.

The protein families discussed above are certainly only a small subset of the repertoire of NHCPs, but perhaps representative of the different modes of action, which range from modifying chromatin "architecture" to acting as targets for other proteins. Most NHCPs are not stably bound to chromatin, but are remarkably mobile within the nucleus.

## Levels of Physical Organization

The large number of variables in chromatin composition have the potential to influence its global and local physical organization, especially the degree of compaction, and the related level of transcriptional competence (higher compaction correlates with reduced transcription). A useful approach is to consider chromatin compaction in terms of hierarchical levels of folding, using a nomenclature based on that devised for proteins (Table I). For chromatin, it is necessary to distinguish between "local" structures that may be confined to specific genes, portions of genes, or groups of genes, and "global" structures that are nucleus wide.

### Global Primary Structures – The Nucleosome Repeat Length (NRL)

While the length of DNA incorporated into the nucleosome is fixed at 146 bp, the length of "linker" DNA between nucleosomes is variable. At the level of the whole nucleus, each cell type in an organism has a characteristic average NRL, most mammalian cells having NRLs between 175 and 195 bp. The longest

**TABLE I**

**Hierarchical Classification Scheme for Chromatin Physical Organization**

| Level of chromatin physical organization | Examples of global features | Examples of local features |
|---|---|---|
| Primary – the linear arrangement of nucleosomes on DNA | The nucleosome repeat length | Preferred locations of nucleosomes and features such as DNase hypersensitive sites on a specific DNA sequence. |
| Secondary – structures formed by interactions of nucleosomes | The "30 nm" chromatin fiber | 3D architecture of nucleosomes and regulatory proteins on a specific DNA sequence. |
| Tertiary – structures formed by interactions between secondary structures | Thicker fibers seen in nuclei and postulated to be composed of 30 nm fibers | Long-distance contacts possibly involving locus control regions, enhancers and promoters, or looped chromatin domains. |
| Quaternary and above – structures formed by interactions between tertiary structures etc. | No unambiguous examples – see text | Interphase chromosome territories; metaphase chromosomes |

NRLs, measured to date, ~240 bp are in echinoderm sperm (which have unusual core and H1 histones), and the shortest, ~165 bp, derived from yeast (*Saccharomyces cerevisiae*), which lack a typical histone H1.

## Local Primary Structures

Overlaying the global NRL for a given nucleus, and the general rule that nucleosomes are randomly deposited, there are many instances where nucleosomes on particular genes and gene-regulatory sequences may be positioned with base-pair precision, and the local NRL may differ from the global NRL. This local positioning often contributes to transcriptional regulation. For example, a nucleosome positioned on a promoter sequence may block the binding of proteins and protein complexes that are required for transcription initiation. Relieving such a transcriptional block may be achieved by a nucleosome "remodeling" process. Other cases are known where a specific DNA sequence is normally nucleosome free. These sites, which are most common in gene-regulatory sequences and are probably occupied by NHCPs *in vivo*, may be detected by their hypersensitivity to nucleases.

Specialized DNA sequences may have a characteristic NRL that differs from the global value. The long stretches of gene-free chromatin at the ends (telomeres) of chromosomes, which consist of tandem repeats of TTAGGG typically have a shorter NRL than bulk chromatin.

Local primary structures may also be influenced by cytosine methylation of CpG dinucleotides, followed by binding of methylated DNA-binding proteins (MBDs). Some MBDs have binding sites for HDAC complexes, leading to local histone deacetylation and hence transcriptional silencing, and there is evidence that MBDs may promote chromatin compaction.

## Global Secondary Structures – The "30 nm" Chromatin Fiber

If chromatin is isolated from nuclei and observed by electron or atomic force microscopy, it is seen to have a conformation highly dependent on the ionic strength of the medium – at low ionic strength (5–25 mM $Na^+$), the linear array of nucleosomes is clearly seen, while at higher ionic strengths, increasing compaction occurs, leading to the formation of an irregular fiber ~30 nm in diameter. This reversible transformation, which is dependent on H1, can also be monitored by changes in physical properties such as sedimentation velocity. Much research effort has been expended on determining the physical organization of the 30 nm fiber, and several different models have been proposed. The most widely discussed are solenoidal structures in which the linker DNA continues the superhelix established in the nucleosome, creating a "stack-of-coins" which subsequently coils into a 30 nm diameter fiber, and zigzag structures in which the linker DNA remains more or less straight and compaction results from the accordion-like compression of the zigzag. Among recent results supporting a zigzag arrangement are computer models derived from force-extension curves of individual chromatin fibers (Figure 1), which bear a striking similarity to native chromatin fibers imaged in the hydrated form (Figure 2). The physical irregularity of native chromatin fibers, which probably results largely from the local variations in NRL has contributed to the ongoing controversy. The ability to reconstitute artificial chromatin with regularly spaced nucleosomes may finally provide an unequivocal answer to the structure of the 30 nm fiber.

## Local Secondary Structures

There is much evidence that local chromatin secondary structures play a large role in chromatin function by promoting or repressing transcription, but little is known of the precise physical organization of these local structures. In some instances, it has been demonstrated that a stretch of chromatin may accumulate many copies of NHCP that acts as a transcriptional silencer (e.g., HP1), but the difficulty of isolating single gene chromatin for observation precludes rapid progress.

## Tertiary and Higher Level Features

Although it is often assumed that native chromatin exists as a series of hierarchical structures above the 30 nm chromatin fiber (and such illustrations are common in textbooks), there is very little solid supporting data. Methods used to examine isolated 30 nm fibers fail to reveal tertiary or higher levels of folding as the ionic

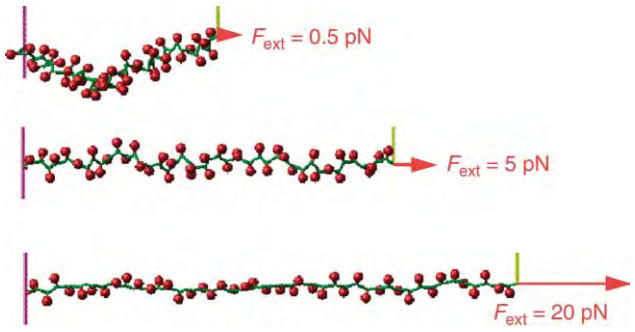



**FIGURE 1** Computer models of chromatin secondary structure at different levels of extension ($F_{ext}$) based on force-extension data derived from pulling single chromatin fibers. Nucleosome core particles are represented by red spheres, and linker DNA by green rods. Modified from Katritch, V., Bustamante, C., and Olson, W. K. (2000). Pulling a single chromatin fiber: Computer simulations of direct physical manipulations. *J. Mol. Biol.* **295**, 29–40.

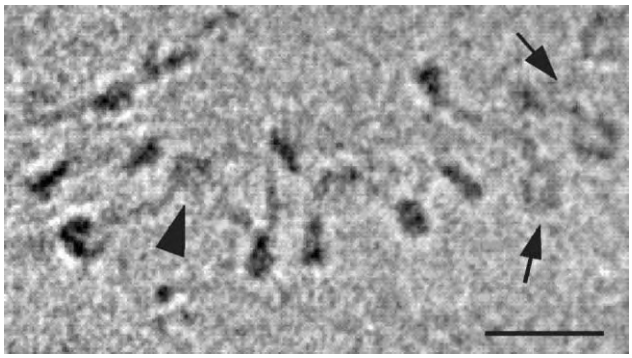


**FIGURE 2** Chromatin fiber observed in the frozen hydrated state in 20 mM NaCl. Many of the nucleosomes are viewed edge-on and appear as dark ovals. Arrowheads point to two nucleosome showing the circular enface profile. The zigzag secondary structure bears a strong resemblance to the model in Figure 1. Scale is 30 nm. From Bednar, J., Horowitz, R. A., Grigoryev, S. A., Carruthers, L. M., Hansen, J. C., and Woodcock, C. L. (1998). Nucleosomes, linker DNA, and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin. *Proc. Natl Acad. Sci. USA* **95**, 14173–14178.

strength is increased toward the "physiological" level. Rather, chromatin tends to aggregate. Examination of thin sections of nuclei in the electron microscope reveals many different "fiber" diameters ranging up to 240 nm, with no clear hierarchical arrangement. Indeed, only under rather specialized conditions and with inactive nuclei can 30 nm fibers be routinely observed in sections. Whether this lack of order *in situ* is due to problems of adequate structural preservation, or simply to a very unstructured and dynamic native organization remains to be seen.

## Chromatin Dynamics

Recent work has revealed that living nuclei are remarkably dynamic. While the nucleosome core histones are long lived and stably incorporated, histone H1 binds to chromatin with a half-life of a few minutes, and most NHCPs have half-lives of a few seconds. Modeling of chromatin fibers based on thermodynamic principals indicates a constant fluctuation in local compaction. Also, individual clusters of genes may change dramatically in compaction state and even location in the nucleus when transcription is stimulated. These observations reinforce the concept of the living nucleus as a highly dynamic entity, both in terms of genetic activity and physical organization.

### See Also the Following Articles:

Chromatin Remodeling • Metaphase Chromosome • Nuclear Organization, Chromatin Structure, and Gene Silencing

### Glossary

**chromatin** Generic term used for (1) material in the nucleus that stains strongly with basophilic dyes and (2) the complex of DNA and chromatin that may be extracted from nuclei for *in vitro* study.

**histone** Family of highly conserved, small basic proteins which constitute the principal protein components of chromatin.

**histone modification** Covalent addition of one or more chemical groups to a specific amino acid in a histone. Modifications include acetylation, methylation, phosphorylation, poly-ADP ribosylation, and ubiquitination.

**histone variant** A histone molecule derived from a different gene and having specific amino acid differences from the "canonical" histone type.

**nonhistone chromatin proteins** Generic term for proteins other than histones that may be bound to chromatin.

**nucleosome** The complete repeat unit consisting of a nucleosome core particle and the linker DNA that continues to the next nucleosome core particle, often with histone H1.

**nucleosome core particle** The disc-shaped (11 nm diameter, 6 nm high) complex of 146 bp of DNA and octamer of histones H2A, H2B, H3, and H4 that is the fundamental structural unit of chromatin. The X-ray structure of the core particle has been solved.

### Further Reading

Allis, C. D. and Jenuwein, T. (2001). Translating the histone code. *Science* **293**, 1074–1080.

Bednar, J., Horowitz, R. A., Grigoryev, S. A., Carruthers, L. M., Hansen, J. C., and Woodcock, C. L. (1998). Nucleosomes, linker DNA, and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin. *Proc. Natl Acad. Sci. USA* **95**, 14173–14178.

Bustin, M. (2001). Chromatin folding and activation by HMGN chromosomal proteins. *Trends Biochem. Sci.* **26**, 431–438.

Katritch, V., Bustamante, C., and Olson, W. K. (2000). Pulling a single chromatin fiber: Computer simulations of direct physical manipulations. *J. Mol. Biol.* **295**, 29–40.

Misteli, T. (2001). Protein dynamics: implications for nuclear structure and function. *Science* **291**, 843–847.

Singh, P. B., and Georgatos, S. D. (2003). HP1: Facts, open questions, and speculation. *J. Struct. Biol.* **140**, 10–16.

Van Holde, K. E. (1988). *Chromatin*. Springer-Verlag, New York.

Wolffe, A. (1998). *Chromatin Structure and Function*, 3rd edition. Academic Press, San Diego, London.

Woodcock, C. L., and Dimitrov, S. (2001). Higher-order structure of chromatin and chromosomes. *Curr. Opin. Genet. Develop.* **11**, 130–135.

### Biography

Christopher L. Woodcock received his bachelors and doctoral degrees at University College London, and pursued postdoctoral research at the University of Chicago and Harvard University before joining the University of Massachusetts Amherst in 1972, where he is now Gilbert Woodside Professor of biology and Chair of the biology department. His interest in chromatin organization began in the early 1970s at the inception of nucleosome model of chromatin, and he has focused his research largely on the physical organization of nucleosomal arrays using direct visualization by electron microscopy as the major analytical tool.

# Chromosome Organization and Structure, Overview

**Elena Gracheva and Sarah C. R. Elgin**
*Washington University, St. Louis, Missouri, USA*

**A chromosome is the packaged form of a single linear double-helical DNA molecule. The genome of a eukaryotic organism consists of from 1 to over 200 chromosomes. Chromosomes are located in the nucleus of the cell and exist in the form of chromatin, a complex between the DNA, the histones (small, highly basic proteins), nonhistone chromosomal proteins (both enzymatic complexes and structural components), and a small amount of RNA (both nascent transcripts and structural components). The numbers and sizes of chromosomes vary considerably between species (*Drosophila* has four chromosomes per haploid cell, human has 23, etc.). As cells enter mitosis, the chromatin is condensed into readily observed, rod-shaped structures, the metaphase chromosomes; a complex process, as described below, is required to package the linear DNA molecule in this form, typically achieving 10,000-fold compactness. Metaphase chromosomes were one of the first subcellular structures observed by cell biologists of the 19th century. Chromosomes were subsequently identified as the vehicle for transmission of genetic information during cell division. As the daughter cells return to interphase, the nucleus reforms, and the chromosomes are decondensed and distributed within the nucleus. The level of DNA packing is much lower during interphase, but is of critical importance in the regulation of gene expression.**

**All genetic information – genes, regulatory elements and signals for structural organization – is arranged linearly along chromosomes. The chromosomes provide not only the means for inheritance, but also the template for transcription, the process of "reading out" the information required by the cell. Different and specific expression profiles are needed for each cell type and stage of development. A full understanding of the mechanisms used in the global regulation of gene expression will not be achieved until the relationships between the linear content of each chromosome, its local and higher-order packaging, or chromatin structure, and the three-dimensional arrangement of the chromosomes in the nucleus have been defined.**

## The Structure of Chromatin

The nucleotide sequence of DNA represents the primary level of chromosome organization. When a DNA molecule is packaged into chromatin, the latter does not behave as a uniform fiber. The chromosome conformation is affected by regulatory and structural features. These sequence elements include promoter proximal elements that control the activity of individual genes, locus control regions that impact a cluster of genes, and boundary elements that potentially regulate the activity of large (10–100 kb) chromosomal domains. In addition to the unique sequences that code for genes, eukaryotic genomes include a high percentage of repetitious DNA, both tandem arrays of short-repeating motifs (often identified as "satellite" DNA because of its different density) and dispersed relics of transposable elements, retroviruses, and the like. Some of the repetitious DNA is utilized in the generation of special chromosome structures such as the centromere, the site of microtubule attachment during mitosis, and the telomeres, structures that protect the ends of the DNA molecules.

The discovery of nucleosomes, repeating subunits based on DNA folding around an octamer of histones, provided a molecular description of the fundamental unit of chromatin folding and accounted for the first six- to sevenfold linear compaction of the DNA. The octamer, containing two molecules each of the core histones H2A, H2B, H3, and H4 (a tetramer of $[H3 + H4]_2$ plus two dimers of [H2A + H2B]) with 147 bp of DNA wrapped around it in 1 2/3 left-handed turns, makes up the core particle. The high-resolution 2.8 Å structure reveals the details of histone organization within the nucleosome (Figure 1). The C-terminal two-thirds of the histone molecules, folded in a "handshake" motif, form the core of the nucleosome, while their amino-terminal tails extend outwards and are potentially involved in further interactions with DNA, with other proteins, and between adjacent nucleosomes. Typically 170 bp of DNA follows a bent path round the histone core, held strongly by electrostatic bonds between the basic amino acids of the histones and the phosphates of the DNA. The segments of DNA that link adjacent nucleosome core particles ("linker" DNA) are bound by linker histones (H1 and its variants) or

 © 2004, Elsevier Inc. All Rights Reserved. 

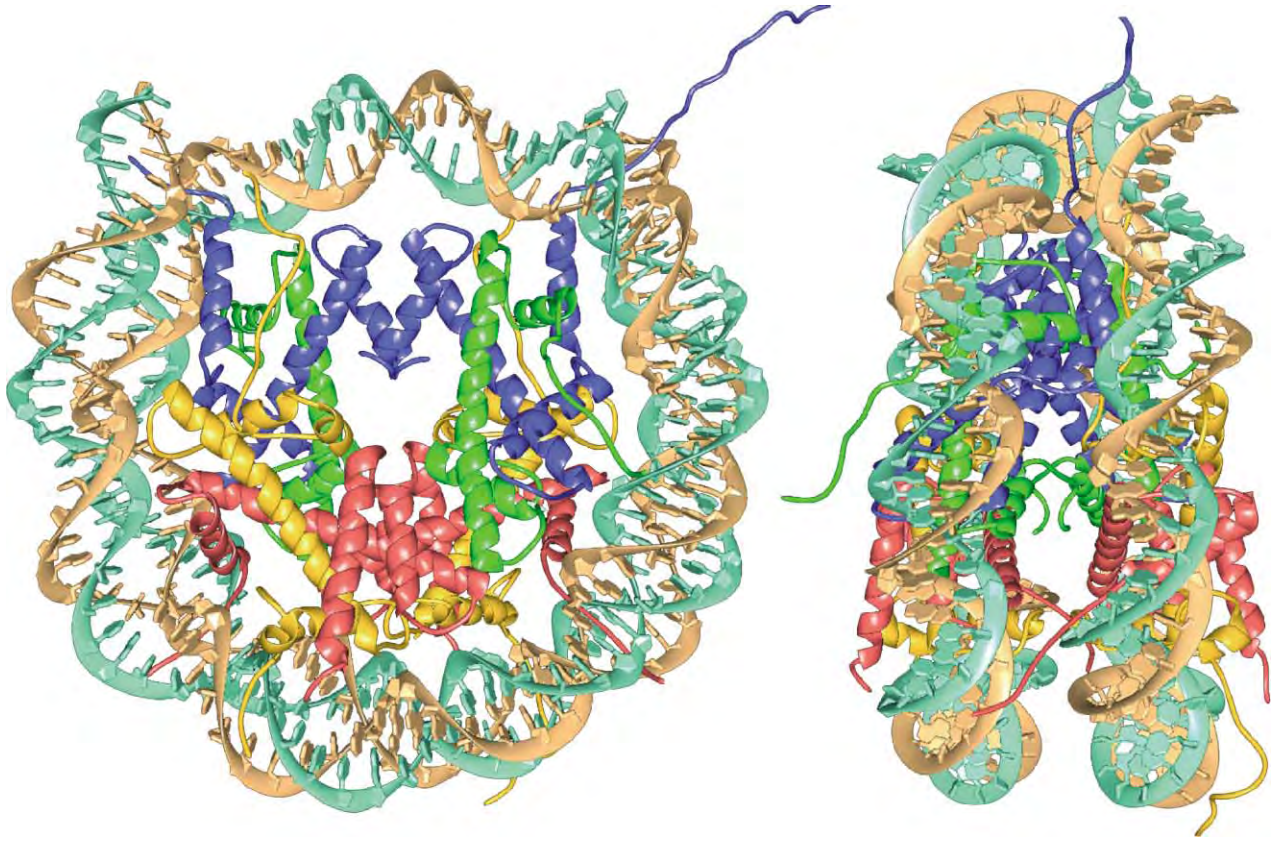


**FIGURE 1** The structure of the nucleosome core particle. Brown and green ribbon traces represent the double-stranded 146 bp fragment of DNA. H2A molecules are shown in yellow, H2B in red, H3 in blue, and H4 in green. (Reprinted from Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8Å resolution. *Nature* **389**(6648), 251–260.)

HMG proteins. These proteins contribute to the further folding of the chromatin fiber, playing a role in the condensation of the 10 nm fiber to form a 30 nm fiber (Figure 2).

The arrangement of nucleosomes within the 30 nm fiber remains largely unknown. A widely utilized model, derived primarily from physical measurements and diffraction studies, is based on a solenoid, in which the nucleosomal fiber is wound in a regular helix, with about six nucleosomes per turn. The linker histones face inwards and help to stabilize the solenoid. However, new single-molecule methods, using microscopy to study single chromatin fibers, suggest a different picture. Both atomic force microscopy (AFM) and cryo-electron microscopy (EM) images show a chromatin fiber that appears as an irregular helix, with a fluctuating zigzag secondary structure. These images do not support the notion of regular solenoids. Comparative studies suggest that the linker DNA with the associated linker histone generates a specific orientation that directs the higher-order folding and compaction of chromatin along the 30 nm fiber axis in an accordion-like manner (Figure 2B).

The 30 nm fiber is thought to be the basic form of much of the chromatin during interphase. However, a subfraction of the chromatin remains condensed, and darkly staining (heteropycnotic), as the cell returns from metaphase to interphase. This condensed fraction is referred to as "heterochromatin," while the more diffuse material is labeled "euchromatin."

Heterochromatic domains contain a high proportion of repetitious DNA sequences (satellite DNA, retrotransposons, and other transposable element relics), have a low gene density, are replicated late in S phase, and show little or no meiotic recombination. Genes normally resident in euchromatin become inactive when placed in or near a heterochromatic environment by rearrangement or transposition. The factors that define specific chromosomal domains as preferred sites of heterochromatin assembly are not well understood, but it appears likely that heterochromatin formation is linked to the presence of repeated DNA sequences. Heterochromatic regions show a more regular nucleosome array (more even spacing) than euchromatic regions; however, the precise nature of the difference in packaging between heterochromatin and euchromatin remains unknown.

Chromatin structure is intimately involved in the regulation of chromosome activities: replication, transcription, and repair. The packaging of a DNA

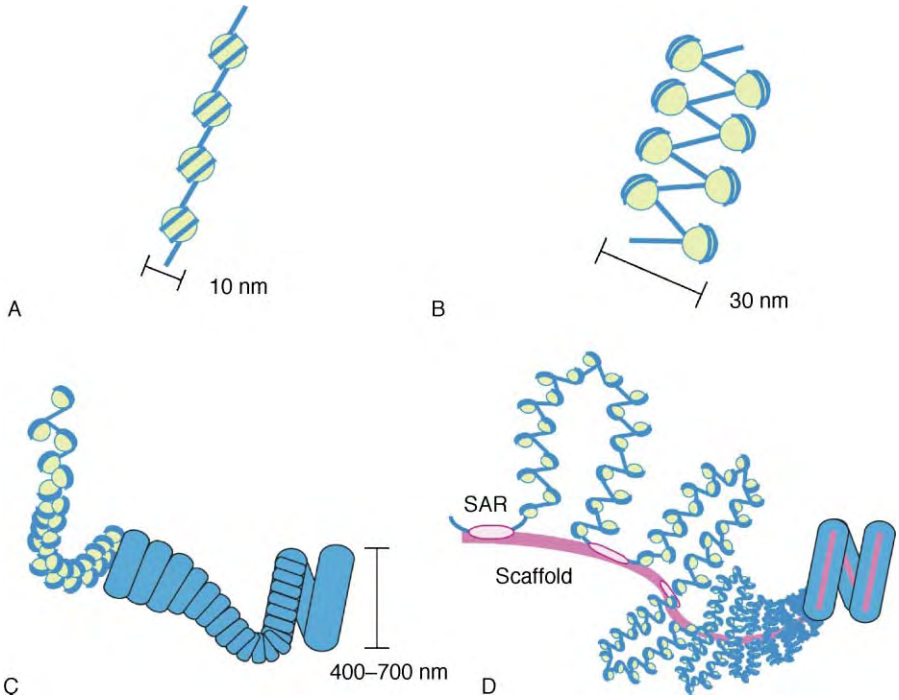



**FIGURE 2** Hierarchical levels of chromatin organization. (A) Structure of the 10 nm nucleosome fiber. Histone octamers are shown as yellow circles, the blue line designates double-stranded DNA wrapped around an octamer. (B) Possible structure of the 30 nm chromatin fiber based on a three-dimensional zigzag arrangement. (C) Formation of higher-order chromatin structure by coiling into thicker (400–700 nm) chromatin fibers. (D) Organization of the chromatin into loops radiating out from a scaffold (magenta). Pink ovals represent SAR elements. (Reprinted from Swedlow, J. R., and Hirano, T. (2003). The making of the mitotic chromosome: Modern insights into classical questions. *Molecul. Cell.* **11**(3), 557–569.)

protein-binding motif with histones in a nucleosome often renders that signal invisible to the protein. For example, packaging of a TATA box sequence into a nucleosome blocks initiation of transcription; assembly of a restriction site into a nucleosome blocks cleavage by that restriction enzyme. Thus, it is no surprise that chromatin needs to undergo significant structural change, or "remodeling," to enable the cellular machines that perform various functions to gain access to their template, the double-helical DNA. The challenge to understand chromatin structure and dynamics, and to link chromatin structure to function, is enormous. Current research has highlighted the role of postsynthetic modifications of both the histones and the DNA in "marking" different structural states and providing appropriate signals to the cellular machines.

The core histone proteins are highly conserved; in the most extreme example, there are only two amino acid changes between the sequences of histone H4 from plants and animals. While the globular carboxy-terminal domains make up the nucleosome core, the flexible amino-terminal tails protrude outward from the core structure. These basic tails function as acceptors for a variety of posttranslational modifications, including acetylation, methylation, and ubiquitination of lysine (K) residues, phosphorylation of serine (S) and threonine (T) residues, and methylation of arginine (R) residues. Several studies have shown that histone modifications can be cooperative or conflicting, one modification facilitating or blocking another. The combination of histone modifications present determines the chromatin status of small and large regions of the genome and mediates its functional activity. In particular, histone acetylation is associated with gene expression, while deacetylation, with methylation of histone H3 on lysines 9 and/or 27, is associated with silencing. These patterns can be altered by multiple extracellular and intracellular stimuli that signal to the nucleus. In fact, recent studies have shown that many proteins characterized as activators or silencers of transcription work by causing or facilitating a change in the modification state of the histones at a given locus. Thus, chromatin itself has been proposed to serve as a signaling platform and to function as a genomic integrator of various signaling pathways. Covalent modifications of histone tails are believed to act in concert to establish the "histone code" essential for assembly of the desired chromatin state.

In addition to the proteins that package DNA, there are also sets of proteins that interact with specific sequences to control gene expression, interacting with promoters, enhancers, locus control regions, etc. While some such proteins can recognize DNA sequences in a nucleosome, many cannot. Both targeted alterations in histone modification and the remodeling of nucleosomes

are often required to allow these latter proteins to function. The order of the required operations for activation of transcription varies at different loci.

Other nonhistone proteins contribute to structural elements such as insulators and/or boundary complexes. Insulators define a region of regulatory activity by blocking enhancer/promoter interactions, while boundaries (or barriers) limit the spread of heterochromatin into a euchromatic domain. The arrangement of these genomic features contributes to the functional design of a locus and region. Unfortunately, it has not yet been possible to relate boundary elements, defined by their functional role described above, with the structural elements that anchor loops of DNA, as seen by electron microscopy, or with the protein scaffold that constrains the DNA in supercoiled domains of 10–100 kb. A better understanding of this level of organization will be essential to develop an understanding of how chromosomes function.

## The Role of Spatial Organization of the Genome in the Nucleus

The next level of chromosome organization must take into account the three-dimensional arrangement of the chromosomes within the cell nucleus. Approaches to microscopy that allow us to examine the nucleus in three dimensions have shown that individual chromosomes occupy defined subdomains within the nucleus. This organization involves the interaction of different factors bound to specific sites in the chromosomes with regions of the nuclear envelope; the protein-facilitated juxtaposition of chromosome sites to form stable connections between distant sequences, with intervening loops of the chromatin fiber; and the possible interaction of certain DNA regions (MARs, or matrix attachment regions) with some nonchromatin elements of internal nuclear structure. Recently, there has been increasing evidence that both actin and myosin, found inside the nucleus, might have an impact on its internal structure. Unfortunately, our understanding of this higher-level packaging is very meager compared to our knowledge of the primary levels of organization. Nonetheless, such packaging can be thought of as the culmination of the hierarchy of chromatin structures that functionally connect the linear genomic sequence to the mechanisms for information processing inside the cell nucleus.

## The Mitotic Chromosome

Substantial differences in the organization of chromosomes occur during mitosis. The most obvious difference is that the chromosome structure is more compact and the shape much better defined than in interphase chromosomes. This more stable structure has offered some advantages for examining the organization and packaging of the single DNA molecule of a chromatid. Several biochemical and structural studies have argued for the partitioning of the genome into a series of defined units (domains) containing 10–100 kb of DNA. Early EM studies suggested the presence of a chromosome scaffold from which loops of chromatin radiate. The scaffold might be considered as either an axial core, presumably made up of DNA and/or protein, or a loose network of DNA/protein complexes whose regulated assembly controls the higher-order structure of the chromosome. In general, scaffold models presume interactions between specific protein factors and *cis*-acting DNA sequences (SARs, or scaffold attachment regions) that determine the limits of the domains. In most models, two neighboring elements, separated by up to 100 kb of DNA, bind to the scaffold forming a loop. Experimentally, a group of very AT-rich sequences has been identified as potential SARs, based on the association of these sequences with the protein scaffold remaining after histones have been extracted from chromosomes. Using fluorescence *in situ* hybridization (FISH), SARs were visualized as a long queue running the length of the chromosome that alternately followed a fairly linear or tightly coiled path. These images were highly variable. Helically folded mitotic chromosomes have been visualized under certain conditions, suggesting that the highest order of folding is a helical winding of the chromosome arm.

However, *cis*-acting elements affecting chromosome architecture have not been identified by genetic criteria, nor have the proposed SARs (identified by biochemical assay) been mapped successfully in relation to cytological preparations. Recently, biophysical approaches for quantifying the elastic properties of mitotic chromosomes have provided important insights into their structure. The mechanical integrity of mitotic chromosomes appears to be maintained by links between chromatin fibers, not by a proteinaceous axial core. Isolation of whole chromosomes and reconstitution of mitotic chromosomes has identified topoisomerase IIa, a five-subunit complex termed condensin, chromokinesin, and the chromatin remodeling ATPase ISWI as major constituents. All presumably contribute to the change from the interphase form to the condensed mitotic chromosome. ATPases are very abundant in mitotic chromosome fractions. Probably these enzymes provide the energy for the aforementioned complexes to induce the local and global conformational changes in chromosomes required to support condensation, and microtubule-dependent movement of assembled chromosomes to opposite poles prior to cell division.

The centromere, observed in all higher eukaryote chromosomes, is a well-known landmark. It takes the form of a distinct primary constriction where the sister chromatids are held together until separated. The constricted region comprises a differentiated chromatin structure onto which microtubules bind to provide proper chromosome movements during the processes of mitosis and meiosis. The centromere in higher eukaryotes is generally made up of massive regions of repetitious sequences. Many observations suggest that there is no specific DNA sequence that is required for centromere formation, strongly implicating an epigenetic mechanism. Five mammalian CENP proteins (CENtromere Proteins) – CENP-A, CENP-B, CENP-C, CENP-G, and CENP-H – are unique and essential to the centromere. Three of these proteins – CENP-A, CENP-B, and CENP-C – have demonstrated DNA-binding activity and are potential candidates for marking the site. CENP-A is a histone H3-like protein that is conserved in mammals and other organisms; it is present only in the nucleosomes of active centromeric regions, where its presence triggers kinetochore formation. A mechanism for centromerization is not identified at present. Most of the chromatin properties of centromeres are not unique. For example, hypoacetylation of histones, a high level of DNA methylation and late replication, are also characteristic features of noncentromeric heterochromatin. The most attractive candidate for the centromeric mark is CENP-A, but the mechanism for incorporation of CENP-A into only one site per chromosome remains a puzzle.

The ends of eukaryotic chromosomes are organized into telomeres, nucleoprotein structures essential for maintaining the integrity of the genome, the so-called "guardians of the chromosome." Telomeric DNA in the majority of eukaryotes is made up of tandem repeats of short guanine-rich sequences. In some cases (e.g., in *Drosophila*), a subset of transposable elements is maintained at the chromosome ends and guanine-rich repeats are not observed. The telomeres show a very special pattern of chromatin fiber organization, distinct from the rest of the genome. The G-rich sequences can form a quadruple helix. *In vivo*, telomeres are not necessarily linear, but can form looped foldback structures, stabilized by protein components. Many of the key proteins in the telomere have been identified, although their functions are still poorly understood.

## Summary

A chromosome, then, can be considered to be a single, double helical DNA molecule, running from one telomere through the centromere to the other telomere. Multiple origins of replication, and many genes, are typically found distributed along the length of the DNA molecule. The DNA is packaged first in a nucleosome array, subsequently in a 30 nm fiber, and finally in higher-order structures. This packaging contributes to gene regulation both at the level of individual genes, and at the level of large domains.

## See Also the Following Articles

Chromatin: Physical Organization • Chromatin Remodeling • Nuclear Organization, Chromatin Structure, and Gene Silencing • Nucleoid Organization of Bacterial Chromosomes • Metaphase Chromosome

## Glossary

**chromatin** The highly dynamic complex of DNA, histones, and nonhistone proteins from which the eukaryotic chromosome is built; it is generally used in reference to the interphase form and can refer to the sum of all chromosomes.

**chromosome** Structural unit of the genetic material in eukaryotes. It consists of one double-stranded DNA molecule, packaged as interphase chromatin or a metaphase chromosome. Eukaryotes have from 1 to over 200 chromosomes, depending on the species.

**nucleosome** Fundamental repeating subunit of the chromatin fiber, consisting of a core histone octamer (made up of two molecules each of H2A, H2B, H3, and H4) with DNA wrapped around the surface in nearly two left-handed turns, connected by linker DNA associated with one molecule of linker histone (usually H1) to the next subunit. Each repeating unit (core plus linker) packages about 200 bp of DNA.

## Further Reading

Fischle, W., Wang, Y., and Allis, C. D. (2003). Histone and chromatin cross-talk. *Curr. Opin. Cell. Biol.* **15**(2), 172–183.

Grewal, S. I., and Elgin, S. C. (2002). Heterochromatin: New possibilities for the inheritance of structure. *Curr. Opin. Genet. Dev.* **12**(2), 178–187.

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8Å resolution. *Nature* **389**(6648), 251–260.

Mellone, B. G., and Allshire, R. C. (2003). Stretching it: Putting the CEN(P-A) in centromere. *Curr. Opin. Genet. Dev.* **13**(2), 191–198.

Neidle, S., and Parkinson, G. N. (2003). The structure of telomeric DNA. *Curr. Opin. Struct. Biol.* **13**(3), 275–283.

O'Brien, T. P., Bult, C. J., Cremer, C., Grunze, M., Knowles, B. B., Langowski, J., McNally, J., Pedesson, T., Politz, J. C., Pombo, A., Schmahl, G., Spatz, J. P., and , von Driel, R. (2003). Genome function and nuclear architecture: From gene expression to nanoscience. *Genome Res.* **13**(6A), 1029–1041.

Summer, A. T. (2003). *Chromosomes: Organization and Function.* Blackwell Publishing, Malden, MA.

Swedlow, J. R., and Hirano, T. (2003). The making of the mitotic chromosome: Modern insights into classical questions. *Molecul. Cell.* **11**(3), 557–569.

West, A. G., Gaszner, M., and Felsenfeld, G. (2002). Insulators: Many functions, many mechanisms. *Genes Dev.* **16**(3), 271–288.

Zlatanova, J., and Leuba, S. H. (2003). Chromatin fibers, one-at-a-time. *J. Mol. Biol.* **331**(1), 1–19.

## Biography

Sarah C. R. Elgin is a Professor in the Department of Biology at Washington University in St. Louis.

Elena Gracheva is a Research Associate in the Elgin lab. The Elgin lab is interested in the role that chromatin structure plays in gene regulation, considering both effects from packaging large domains and local effects of the nucleosome array. Biochemical, genetic, and cytological studies are conducted using *Drosophila melanogaster* as a model organism. Ongoing projects include investigating the mechanisms of gene silencing associated with heterochromatin formation, and investigating the relationship between heterochromatin and euchromatin. Dr. Gracheva is also involved in investigation of the role of GAGA factor in transcription regulation.

# Coenzyme A

**M. Daniel Lane**
*Johns Hopkins University School of Medicine, Baltimore, Maryland, USA*

**Coenzyme A (CoA) functions as a cofactor for numerous enzyme-catalyzed reactions in animal, plant, and microbial metabolism. The structure of CoA shows that it is composed of the B-vitamin, i.e., pantothenic acid, β-mercaptoethylamine, and an adenine nucleotide with both a 5′-pyrophosphate and a 3′-monophosphate.**

**Most carboxylic acids, e.g., fatty acids, must be "activated" to enter metabolic pathways by conversion to their corresponding acyl–S–CoA (R–CO–S–CoA) thioesters. The sulfhydryl group (–SH) of the β-mercaptoethylamine component of CoA is the site at which carboxylic acids (R–$CO_2H$) bond to form acyl–thioester derivatives. Thioesters are the only form in which CoA is known to function in biological systems. Many of the enzyme-catalyzed reactions in which carboxyl groups (–$CO_2H$) undergo carbon/carbon-, carbon/nitrogen- or carbon/sulfur-bond formation utilize acyl–S–CoA thioesters as the acyl donor substrate.**

## Background and Discovery

CoA was discovered by Fritz Lipmann and his colleagues in the early 1950s. The coenzyme was first isolated from large quantities of pig liver extract as the factor required for the acetylation of sulfanilamide, the assay system used to track CoA during its purification. The discovery of CoA and the characterization and determination of its structure (Figure 1) led to Lipmann being awarded the 1953 Nobel prize in Physiology or Medicine. Lipmann's findings opened the door for the discovery of the innumerable roles of CoA, most notably the discovery by Feodor Lynen that "active acetate" was acetyl–CoA, a key intermediate in the metabolism of carbon compounds by all organisms. In 1964, Lynen was awarded the 1964 Nobel prize in Physiology or Medicine for his discovery of acetyl–CoA and of the many metabolic systems that CoA functions. We now know that CoA plays a key role in carbohydrate, lipid, and amino acid metabolism.

## Enzymatic Functions of CoA and 4′-Phosphopantetheine

### Chemical Mechanisms of Action

CoA occurs in cells either as free CoA or in the form its acyl–S–CoA thioester or as 4′-phosphopantetheine (4′-PP), which is part of a multifunctional enzyme. The sulfhydryl (–SH) group of the coenzyme is the functional site that endows it with its unique chemical/enzymatic properties. In all of the reactions in which it participates, CoA serves to "activate" carboxyl groups (R–CO–OH) in the form of an acyl–CoA thioester derivative (R–CO–S–CoA). In this form the acyl group has favorable energetic and mechanistic properties. Energetically, CoA thioesters have high phosphoryl transfer potential (i.e., a high negative free energy change associated with breaking the C–S bond of the thioester) that facilitates driving the acyl transfer reaction in which it participates toward completion. Mechanistically, the chemistry of CoA thioesters facilitates reactions either at the carbonyl carbon (–$CH_2$–CO–S–CoA) or the adjacent α-carbon atom (–$CH_2$–CO–S–CoA).

#### "Activation" of the Carbonyl- and α-Carbon Atoms in Acyl–CoA Thioesters

All enzymatic reactions of CoA thioesters in metabolism involve "activation" either of the carbonyl-carbon or the adjacent α-carbon atom for facile chemical reaction. Mechanistically, the –CO-carbon (i.e., R–$CH_2$–CO–S–CoA) of the acyl group reacts with a nucleophilic acceptor molecule ('$R^-$; see Figure 2, Rx′n 1) or the adjacent alpha carbon (i.e., R–$CH_2$–CO–S–CoA) reacts with an electrophile ('$R^+$; see Figure 2, Rx'n 2) to produce a new carbon–carbon bond. These reaction types are illustrated below.

### CoA as Donor of 4′-Phosphopantetheine for Fatty Acid Synthase

Fatty acid synthase (FAS) is a large multifunctional enzyme that catalyzes all steps in fatty acid synthesis, catalysis being facilitated by a covalently linked 4′-phosphopantetheine (4′-PP) prosthetic group. The 4′-PP group, which is derived from CoA (Figure 1), is enzymatically transferred to the FAS apoenzyme where it becomes covalently linked to the enzyme. The 4′-PP prosthetic group acts as a long sidearm to which intermediates of the pathway are covalently linked. The 4′-PP sidearm allows translocation of the growing

 © 2004, Elsevier Inc. All Rights Reserved. 

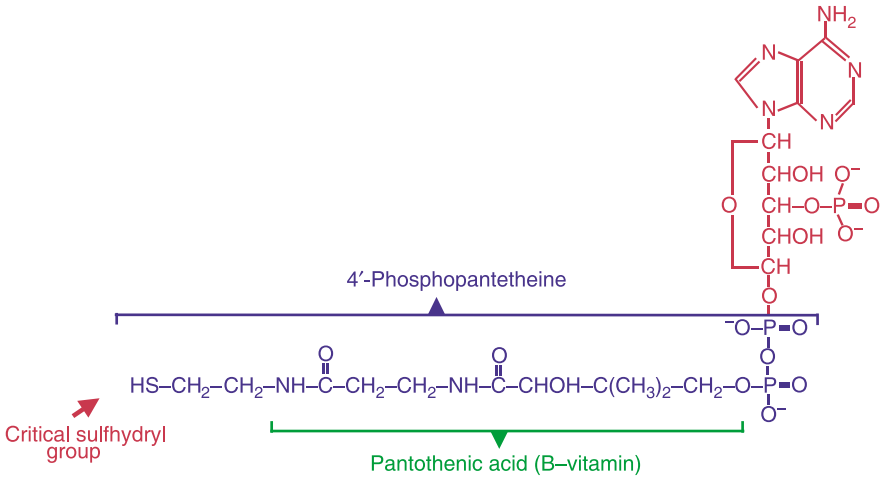



**FIGURE 1** Structure of CoA.

fatty acyl chain intermediate from one catalytic site on FAS complex to the next in the cyclic sequence, this leads to formation of a long-chain fatty acid.

## Enzymatic Synthesis of Acyl–S–CoA Derivatives from Free Carboxylic Acids and CoA–SH

The enzymatic reactions by which acyl–S–CoA derivative is synthesized from carboxylic acids, such as free fatty acids, occur via a common mechanism (Figure 3).

The reaction illustrated uses fatty acid as example; however, it should be noted that virtually all acyl–CoA's are formed from their cognate carboxylic acids by the same mechanism. The enzymes that catalyze these reactions are referred to as acyl–CoA synthetases. In the initial step (Rx′n 1) the fatty acid attacks the $\tilde{\alpha}$ (innermost) phosphate of ATP releasing pyrophosphate ($PP_i$) forming an enzyme-bound acyl–AMP intermediate. In the second step (Rx′n 2), CoA–SH attacks the acyl–CO–carbon of the intermediate producing an acyl–S–CoA product and AMP.

## Metabolic Roles/Functions

CoA and its derivatives function in a wide variety of metabolic pathways including the tricarboxylic cycle, fatty acid oxidation, ketogenesis, fatty acid biosynthesis, sterol synthesis, complex lipid synthesis, amino acid metabolism, and porphyrin synthesis. (Since most of

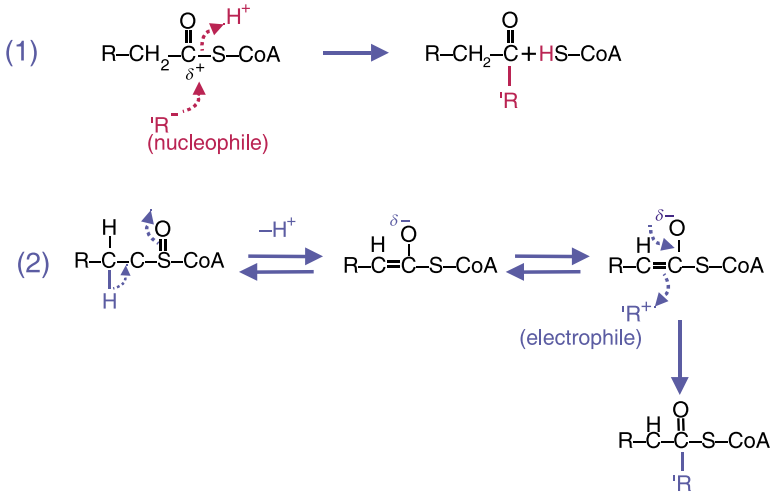



**FIGURE 2** Mechanisms of "activation" of the acyl carbonyl- and $\alpha$-carbon atoms in CoA thioesters.

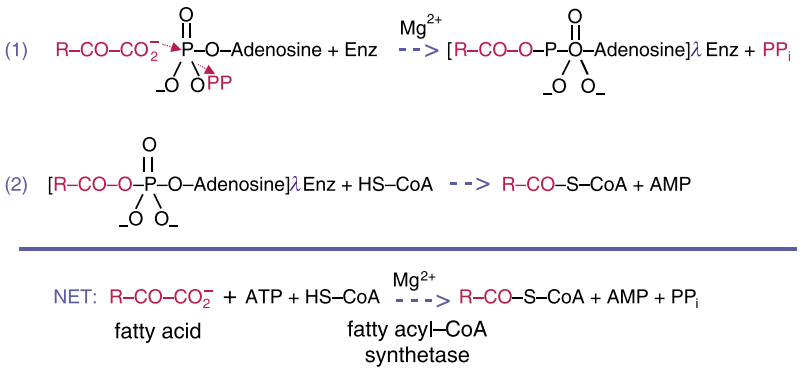



**FIGURE 3** Enzymatic synthesis of acyl–S–CoA's.

these are covered elsewhere in this encyclopedia, they are not dealt with in this entry.) As the fatty acid synthase system is unique, in that it makes use of only a fragment of the CoA molecule, 4′-PP, a brief discussion of FAS is warranted.

## Fatty Acid Synthase

Fatty acid synthesis in animals is catalyzed by a single large (molecular weight, $\sim 5 \times 10^5$) multifunctional enzyme. All eight steps and thus all eight catalytic centers that carry out fatty acid synthesis occur with the intermediates tethered to the FAS. The intermediates are covalently linked by thioester bonds to the –SH group of the long 4′-PP sidearm which facilitates translocation of intermediates from one catalytic center to the next in sequence until the multiple steps of long-chain fatty acid synthesis are completed. Each round of elongation lengthens the chain by two carbons, a process that is repeated 7 or 8 times for the synthesis of a 16- or 18-carbon containing fatty acids.

The process is initiated by the transfer of an acetyl group to 4′-PP from acetyl–S–CoA. The acetyl group linked to 4′-PP serves as the "primer" onto which the long-chain fatty acid is built. Malonyl units from malonyl–CoA, which serve as the chain-elongating group, condenses with the acetyl-primer concomitant with decarboxylation to produce a 4-carbon intermediate that then undergoes two reductive and "1" dehydration step. Successive malonyl groups are transferred to FAS from malonyl–CoA to provide the basic units for successive steps in the elongation process. The terminal step is catalyzed by a thioesterase releasing the long-chain fatty acid product.

## See Also the Following Article

Fatty Acid Synthesis and its Regulation

## Glossary

**coenzyme** A small molecule (molecular weight usually $\leq 1000$) that participates chemically in a reaction catalyzed by an enzyme and upon which the reaction depends.

**electrophile** A compound with an electron-deficient atom that can accept an electron pair from a nucleophilic compound with an electron-rich atom.

**nucleophile** A compound with an electron-rich atom having an unshared electron pair that can be contributed to an electron-poor atom of an electrophilic compound.

**thioester** The condensation product of a carboxylic acid and a thiol (–SH) group formed by removal of a water molecule, e.g.,

$$R\text{–}CO_2H + Co\text{–}SH \rightarrow H_2O + R\text{–}CO\text{–}S\text{–}CoA.$$

## Further Reading

Berg, J., Tymoczko, J. L., and Stryer, L. (2002). *Biochemistry*, 5th edition, W. H. Freeman and Co., New York.

Lipmann, F. (1953). Development of the acetylation problem: A personal account, Nobel Prize Lecture (in Physiology or Medicine). Available on the Nobel Prize Web Site http://www.nobel.se/ or http://www.nobel.se/medicine/index.html.

Lynen, F. (1964). The pathway from "activated acetic acid" to terpenes and fatty acids. Nobel Prize Lecture (in Physiology or Medicine). Available on the Nobel Prize Web Site http://www.nobel.se/ or http://www.nobel.se/medicine/index.html.

Nelson, D. L., and Cox, M. M. (2005). *Lehninger: Principles of Biochemistry*, 4th edition, W. H. Freeman and Co., New York.

Walsh, C. (1979). *Enzymatic Reaction Mechanisms*. W. H. Freeman and Co., San Francisco.

## Biography

M. Daniel Lane is a Distinguished Service Professor in the Department of Biological Chemistry at the Johns Hopkins University School of Medicine. He is a member of the National Academy of Sciences, the American Academy of Arts and Science and has served as President of the American Society of Biochemistry and Molecular Biology. For 19 years he served as Director of the Department of Biological Chemistry at the Johns Hopkins University School of Medicine. His current research interests are on stem cell commitment to the adipocyte lineage, adipocyte differentiation, and the hypothalamic control of food intake, and obesity.

# Collagenases

**Kenn Holmbeck and Henning Birkedal-Hansen**
*National Institutes of Health, Bethesda, Maryland, USA*

**Collagenases are protein capable of cleaving native collagen under physiological conditions *in vivo* and *in vitro*. Here we discuss in brief the history of collagenases, their properties and the definitions assigned to proteins with collagenolytic properties. While collagen cleaving potential is often assessed by simple enzyme substrate incubations it has become increasingly evident that conclusions based on such assay conditions may not necessarily reflect the true nature of enzyme properties. The recent advances in genetic research has demonstrated that some but not all collagen cleaving enzymes functions in that capacity *in vivo*. Conversely, other enzymes previously regarded as unrelated to collagenases have been proven to be important for collagen metabolism.**

## What Is a Collagenase?

The term "collagenase" was first used by Gross and Lapiere in their 1962 description of an activity released from cultured tissue explants of involuting tadpoles, which dissolved otherwise highly proteinase-resistant, reconstituted collagen fibrils. This was a groundbreaking finding. It was the first demonstration of the existence of an activity operating at neutral pH that was fully capable of dissolving and cleaving native collagen fibrils. It marked the beginning of an exciting period of discovery that led to identification of a large number of distinct matrix metalloproteinases (MMP), almost 6–7 in number with collagen-cleaving capabilities under various *in vitro* conditions (Table I).

## Original Definition of Collagenases

The unique resistance of native collagen molecules to proteolysis is a result of the tightly wound, semirigid triple helical structure. However, the resistance is only relative and not absolute. Generally, one of two tests was applied to ascertain whether an enzyme or an activity represented a true collagenase: (1) ability to dissolve reconstituted type I collagen fibrils in the temperature range 35–37°C, and/or (2) ability to cleave type I collagen in solution at neutral pH in the temperature range 15–25°C into two distinct and recognizable 1/4–3/4 fragments (classical collagenase signature cleavage pattern). That the 3/4–1/4 cleavage site is actually utilized *in vivo* was elegantly shown by Robin Poole and co-workers by neo-epitope immunochemistry which convincingly identified collagen molecules in several tissues, which were cleaved at this particular site. Since a number of collagenases cleave this site, the mere detection of this neo-epitope does not necessarily identify any single enzyme responsible for the cleavage. Despite this, the 3/4–1/4 cleavage products are highly useful hallmarks in the analysis and characterization of collagenase activity.

Several lines of evidence support the notion that the collagenase-sensitive cleavage site is subject to local unwinding and therefore constitutes a site of minor resistance. For example, type III collagen has an Arginine-substitution in the susceptible region, and is readily cleaved by trypsin producing a 3/4–1/4 pattern. However, the collagenase-sensitive site in the alpha 1 chain of type I collagen can be mutated in mice without yielding an acute collagen "indigestion" phenotype. The modification leads to a progressive fibrosis, but at a rate indicating that the collagen molecule is cleaved with some efficiency elsewhere. The term "collagenase" based on site specificity may thus be only partially applicable and may in effect obscure the fact that collagen is cleaved at other sites by collagenases, which do not qualify for this designation based on the criteria of generating 3/4–1/4 specific cleavage products.

## Revised Definition of "Collagenases"

Based on the observations outlined above, the original definition of a collagenase will therefore have to be revised and broadened to also accommodate other proteinases such as cathepsin K, which clearly is a potent collagen-cleaving enzyme and yet utilizes other cleavage sites in the triple helical region and functions at acidic pH. Ability to cleave native type I collagen in solution within the temperature range 15–25°C at either

**TABLE I**

**Fibrillar Collagenases (Proteinases with Activity Against Fibrillar Collagens)**

| Enzyme | Other name | Classification | Species |
|---|---|---|---|
| MMP-1 | Collagenase-1, interstitial collagenase | Matrix metalloproteinase | Widely expressed except in rodents |
| McollA | | Matrix metalloproteinase | Mice |
| MMP-2 | Gelatinase, type IV collagenase | Matrix metalloproteinase | Widely expressed |
| MMP-8 | Collagenase-2, PMN collagenase | Matrix metalloproteinase | Widely expressed |
| MMP-13 | Collagenase-3, interstitial collagenase | Matrix metalloproteinase | Widely expressed |
| MMP-14 | MT1-MMP | Matrix metalloproteinase | Widely expressed |
| MMP-18 | Collagenase-4 | Matrix metalloproteinase | Frog |
| Cathepsin K | | Acidic thiol proteinase | Widely expressed |

neutral or acidic pH, or completely dissolve native or reconstituted collagen fibril matrices at 35–37°C still appear to be valid requirements. It is now increasingly being recognized that type I collagen-cleaving ability *in vitro* does not necessarily mean that the enzyme in reality functions as a collagenase *in vivo*. In fact, the assay conditions ordinarily used to assess the collagenolytic activity of putative collagenases may rarely, if ever, entirely mimic the conformation and configuration of the substrate when it is associated with multiple other components of the extracellular matrix and packed to densities rarely achieved in a cell-free system. Conversely, an argument may also be made that enzymes displaying little collagenolytic activity *in vitro* may conceivably be potent collagenases *in vivo*, if by association with other ECM macromolecules, the collagen molecules are made more readily available for cleavage. Standard enzyme kinetic assays measuring substrate conversion are often ill-suited to account for reactions, which take place in a two-dimensional, diffusion-restricted environment such as a cell-membrane or a solid-substrate surface. For collagenolytic enzymes with *trans*-membrane spanning domains, a solution-based assay obviously can change the basic environmental properties under which a molecule ordinarily works, but also molecules commonly recognized as secreted exert their function in close proximity to the peri-cellular (solid phase) environment. Second, the actual enzyme-to-substrate ratio *in vitro* may not adequately mirror that which exists *in vivo*. It may therefore be necessary to revise the definition "collagenase" and assign the term only to enzymes for which there is some evidence, for instance, from naturally occurring mutations or gene ablation studies, that they truly function to cleave collagen *in vivo*.

## The Collagenases

Within a few years of the discovery of the tadpole enzyme, similar activities were identified in many vertebrate species including humans. Among these were an enzyme from PMN leukocytes that later turned out to be a genetically distinct collagenase (MMP-8). Gelatinase A/type IV collagenase (MMP-2) was also identified early on and recognized as a probably distinct and separate activity because of its ability to cleave type IV collagen and gelatin. However, it was not recognized until Aimes and Quigley's important study in 1995 that MMP-2 can also be a genuine type I collagen-specific collagenase once the inhibitor TIMP-2, with which it is often complexed, is removed. This finding raises the question whether, and under what conditions, TIMP-2-free proMMP-2 exists *in vivo*? MMP-13 or collagenase-3 is found in both humans and rodents and displays partial homology to the human interstitial collagenase, MMP-1. It was thus originally thought to be the rodent orthologue of MMP-1, but turned out to be a genetically distinct collagenase capable of also dissolving the otherwise highly resistant and slowly cleaved type II collagen. Surprisingly, rodents appear to express MMP-13 in most places where MMP-1 is expressed in humans, thus suggesting that it is a functional equivalent. Yet a closer rodent orthologue of human MMP-1 called Mcol-A has been identified, but so far has been characterized only to some extent. Based on the initial analysis Mcol-A appears to have some collagen-cleaving ability against type I collagen although the specific activity seems fairly low. A collagenase (MMP-18), which was distinct from MMP-1, MMP-13, and MMP-8, was discovered in the frog but no vertebrate orthologue has been identified. The first membrane-type MMP (MT1-MMP or MMP-14) was discovered in 1994 and shortly after, it was shown to have collagen-cleaving activity at least *in vitro*. Later five additional membrane-bound MMPs were identified but it is not yet known whether they possess collagen-cleaving activity. Although most of the collagenases mentioned so far have been matrix metalloproteinases, it is, as previously mentioned, necessary today to also include the thiol cathepsin, cathepsin K. This potent collagen-cleaving enzyme is utilized in the phagocytic pathway of collagen

degradation and in osteoclastic bone resorption, when mineralized type I or type II matrices are dissolved. The various collagenases display somewhat varying substrate preference against type I, II, and III collagens. Type I collagen is most readily cleaved by MMP-8 and MMP-14, while MMP-1 shows preference for type III collagen and MMP-13 for type II collagen.

## Recent New Insights

The past decade has dramatically altered our view of the role of classical collagenases in normal growth and development. The advent of gene ablation techniques in combination with various mouse models has enabled detailed exploration of the biologic function of the individual collagenases *in vivo*. Unfortunately, MMP-1 cannot be subjected to this approach because it is not expressed in mice and no human mutations of MMP-1 have been characterized. Although a potent collagenolytic enzyme *in vitro*, the true role of MMP-1 *in vivo* may therefore not be known until a human null-mutation is discovered. No reports have emerged yet with regard to ablation of the weakly collagenolytic, putative murine MMP-1 orthologue, Mcol-A. MMP-2 has been knocked out, but the phenotype is very mild when considering that this enzyme is perhaps the most widely expressed MMP. It was subsequently shown that primary tumor mass and metastatic spread was significantly reduced in MMP-2-deficient animals in two independent tumor models, but it is uncertain whether and/or how these observations relate to collagen degradation. So, at this point there is only limited evidence that MMP-2 indeed serves as a collagenase in mice. Adding confusion to this scenario, however, is the finding by Martignetti and co-workers of the first human MMP-mutation (in MMP-2), which yields a rather spectacular generalized osteolytic bone phenotype. It is dramatically different from that of the mouse but it does have features of a collagen indigestion phenotype. Recently, ablation of the PMN collagenase MMP-8 was reported. Interestingly, MMP-8 deficiency, particularly in male mice, resulted in a dramatic rise in DMBA/TPA-induced skin tumors, diminished inflammatory response, and chemokine processing suggesting that an important protective role, conferred by MMP-8, had been lost. It is so far unresolved whether this effect is a function of the loss of collagenolytic activity by PMNs. An MMP-13-deficient mouse has been generated which shows widening of the growth plate presumably as a result of impairment of cleavage of collagen type II in this area. Moreover, osteoclast differentiation and activity is impaired (S. M. Krane, personal communication) and bone formation is increased. The phenotype is thus consistent with MMP-13 serving as a collagen-cleaving enzyme *in vivo*. MT1-MMP- (MMP-14-) deficient mice display a profound and complex phenotype in which growth, development, and maintenance of multiple collagen-containing matrices (bone, skin, tendon, joints, and ligaments) are severely impaired due to loss of an indispensable and uncompensated collagenolytic activity. Since the MT1-MMP knockout phenotype does not resemble those of either MMP-2 or MMP-13, it is highly unlikely that MT1-MMP functions primarily through activation of these enzymes as has been suggested. Rather, MT1-MMP appears to serve as a highly effective collagenase in its own right *in vivo*. However, it is interesting to note that MT1-MMP displays somewhat weaker collagenolytic activity *in vitro* than, for instance, MMP-1. This finding again points to some level of disconnect between collagenolytic capacity *in vivo* and *in vitro*. In the case of cathepsin K deficiency, the predictions of collagenolytic impairment, however, do hold up *in vivo*. Mouse and human cathepsin K gene mutations give rise to pycnodysostosis in humans and osteopetrosis in the mouse consistent with impairment of osteoclast-mediated type I collagen cleavage. These findings help identify cathepsin K as the acidic collagenase required for removal of type I and type II collagens from mineralized matrices, which can only be dissolved in an acidic environment. It is highly likely that cathepsin K also is responsible for the cleavage of soft tissue collagen fibrils in phago-lysosomes during the often-overlooked phagocytic pathway.

## Conclusion

In the aggregate, recent findings have demonstrated that true "collagenases" as defined by their substrate specificity *in vivo* can be found in at least two different classes of proteolytic enzymes (matrix metalloproteinases and cathepsins). Moreover, it is now evident that not all predictions based on enzyme kinetics obtained *in vitro* hold up *in vivo*. Collagenases work in the complex environment of an extracellular matrix. Fortunately, the tools for characterization of enzyme substrate interactions have now entered an exciting new era, which offer multiple genetic approaches to elucidation of these questions. Based on such approaches we have already come to realize that seemingly identical enzymes can perform different roles depending on the species. With this knowledge in mind, the understanding of and search for collagen metabolizing enzymes should continue.

### See Also the Following Articles

Collagens • Metalloproteinases, Matrix

## Glossary

**cathepsins** Lysosymal proteinases, mostly thiol-dependent proteinases of the papain family. Present in multiple forms of which cathepsin K seems to be the most potent collagen-cleaving enzyme.

**collagen** Major structural proteins of the body with type I being the most abundant protein in soft connective tissue and in bone. The major fibrillar collagens (types I, II, and III) provide much of the structural framework for soft connective tissues, cartilage, and bone. They are generally highly resistant to proteolysis.

**collagenase** Enzyme capable of cleaving native collagen under specified conditions (temperature and pH).

**knockout mouse** Mouse rendered deficient for a selected gene by targeted inactivation of the encoding locus in embryonic stem cells, subsequent transmission of the trait to the germ line and breeding of the trait to a homozygous state.

**matrix metalloproteinases (MMPs)** A group of zinc endopeptidases (metzincins) characterized by zinc binding VAAHEXGHXXGXXH amino acid consensus sequence in the catalytic domain and activation by a so-called cysteine switch. This residue is harbored in the latency-conferring prodomain that is proteolytically cleaved upon activation. This group of enzymes does not include the ADAMS, which contains a characteristic disintegrin domain.

## Further Reading


Balbin, M., Fueyo, A., Tester, A. M., Pendas, A. M., Pitiot, A. S., Astudillo, A., Overall, C. M., Shapiro, S. D., and Lopez-Otin, C. (2003). Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat. Genet.* **35**(3), 252–257.

Billinghurst, R. C., Dahlberg, L., Ionescu, M., Reiner, A., Bourne, R., Rorabeck, C., Mitchell, P., Hambor, J., Diekmann, O., Tschesche, H., Chen, J., Van Wart, H., and Poole, A. R. (1997). Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J. Clin. Invest.* **99**, 1534–1545.

Brinckerhoff, C. E., and Matrisian, L. M. (2002). Matrix metalloproteinases: A tail of a frog that became a prince. *Nat. Rev. Mol. Cell Biol.* **3**, 207–214.

Egeblad, M., and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* **2**, 161–174.

Everts, V., Hou, W. S., Rialland, X., Tigchelaar, W., Saftig, P., Bromme, D., Gelb, B. D., and Beertsen, W. (2003). Cathepsin K deficiency in pycnodysostosis results in accumulation of non-digested phagocytosed collagen in fibroblasts. *Calcif. Tissue Int.* **73**, 380–386.

Garnero, P., Borel, O., Byrjalsen, I., Ferreras, M., Drake, F. H., McQueney, M. S., Foged, N. T., Delmas, P. D., and Delaisse, J. M. (1998). The collagenolytic activity of cathepsin K is unique among mammalian proteinases. *J. Biol. Chem.* **273**(48), 32347–32352.

Hotary, K. B., Allen, E. D., Brooks, P. C., Datta, N. S., Long, M. W., and Weiss, S. J. (2003). Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. *Cell* **114**, 33–45.

Krane, S. M., Byrne, M. H., Lemaitre, V., Henriet, P., Jeffrey, J. J., Witter, J. P., Liu, X., Wu, H., Jaenisch, R., and Eeckhout, Y. (1996). Different collagenase gene products have different roles in degradation of type I collagen. *J. Biol. Chem.* **271**(45), 28509–28515.

Liu, X., Wu, H., Byrne, M., Jeffrey, J., Krane, S., and Jaenisch, R. (1995). A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. *J. Cell Biol.* **130**(1), 227–237.

Saftig, P., Hunziker, E., Wehmeyer, O., Jones, S., Boyde, A., Rommerskirch, W., Moritz, J. D., Schu, P., and von Figura, K. (1998). Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc. Natl Acad. Sci. USA* **95**, 13453–13458.

Woessner, J., and Nagase, F. H. (eds.) (2000). *Matrix Metalloproteinases and TIMPs*. Oxford University Press, Oxford.

Zucker, S., and Chen, W. (eds.) (2003). *Cell Surface Proteases*. Academic Press, San Diego.


## Biography

Henning Birkedal-Hansen is a Scientific Director and Senior Scientist at The National Institute of Dental and Craniofacial Research (NIDCR) at the NIH in Bethesda, Maryland. He also leads the Matrix Metalloproteinase Unit that focuses on the remodeling of collagen fibrils in the extracellular matrix and the biologic function of matrix metalloproteinases and related enzymes. He holds a D.D.S. and a Ph.D. from the Royal Dental College of Copenhagen, Denmark. He served on the faculty of the University of Alabama School of Dentistry in Birmingham, Alabama in various positions from 1979 before becoming Scientific Director at the NIDCR in 1994.

Kenn Holmbeck is a Senior Staff Scientist in the Matrix Metalloproteinases Unit. He holds a Ph.D. from the University of Copenhagen, Denmark, and received postdoctoral training at Children's Hospital Research Foundation, Cincinnati, Ohio and the National Institute of Dental and Craniofacial Research.

# Collagens

**Darwin J. Prockop**
*Tulane University Health Sciences Center, New Orleans, Louisiana, USA*

**Collagens are a large family of proteins widely distributed in nature with a simple, repetitive sequence of amino acids that serves as a defining signature for the proteins. The inherent properties of the signature sequence of amino acids drive spontaneous self-assembly of three similar chains into a distinctive triple-helical structure. The long triple-helical structures found in some collagens drive spontaneous self-assembly of the proteins into large fibrils. The shorter triple helices in other collagens provide rigidity to the proteins to complement the biological activity of other functional domains.**

## The Signature (Gly-XY-)$_n$ Sequence of Collagens

In the signature amino acid sequence of collagens, every third amino acid is glycine, the amino acid following glycine is usually the five-member ring amino acid proline, and the next amino acid is hydroxyproline. Therefore the signature sequence is usually depicted as (Gly-XY)$_n$ in which the X position is frequently proline and the Y position is frequently hydroxyproline. Under physiological conditions, three chains with the signature (Gly-XY-)$_n$ sequence spontaneously associate into a three-stranded helix that is unique to collagens. The subclass of collagens that form large fibrils consists almost entirely of long (Gly-XY-)$_n$ sequences tightly coiled into triple-helical structures referred to as collagen monomers. The long triple-helical monomers, in turn, spontaneously associate into long, highly ordered fibrils. In some tissues, the long fibrils associate laterally into thick fibers. The two steps in which (Gly-XY-)$_n$ sequences assemble into triple-helical monomers and the triple-helical monomers assemble into fibers resemble the crystallization of small molecules (Figures 1A and 2). Each step is entropy driven by loss of surface water as the larger structure is formed, much as is seen in many crystallization processes. Also, as with crystallizations, each step involves first the slow formation of a small nucleus of a defined structure, and then rapid propagation of the nucleus to form a large structure in which the molecular architecture of the nucleus is extensively replicated. Glycine, the smallest amino acid, must be in every third position in the collagen triple helix because it occupies a very small space where the three chains come together. Additionally, the hydrogens on the $\alpha$-carbon of glycine allow it to form hydrogen bonds across the three chains. The closed-ring structures of the proline and hydroxyproline residues limit rotation of the polypeptide backbone, thereby providing rigidity to the structure.

Hydroxyproline residues, which are rarely found in other proteins, play a special role in the triple helix. The hydroxyl groups lock the slightly flexible ring of the amino acid into a conformation that further stabilizes the polypeptide backbone. Charged amino acids that occupy the X and Y positions in the repetitive (Gly-XY-)$_n$ sequences point out from the center of the triple helix. The orientation of the charged groups allows them to form salt bridges that bind each triple-helical monomer to surrounding monomers. Most of the monomers in fibrils are staggered one-quarter of their length relative to their nearest neighbors with short gaps between their ends. The gaps are readily seen by staining the surface of the fibrils and examining them by electron microscopy: each fibril has a cross-striated pattern in which the distances between the cross-striations correspond to about one-quarter of the lengths of the monomers. After assembly of the fibrils, the structures are further stabilized by a cross-linking enzyme that generates highly reactive aldehydes by deaminating some of the terminal amino groups on lysine residues that project out from the surface of the monomers. The aldehydes then spontaneously form covalent bonds that link most of the monomers throughout the width and length of the fibril. The result is a highly ordered structure with about the same strength as a steel wire. In essence, the signature (Gly-XY-)$_n$ sequences contain essentially all of the information and driving forces needed to spontaneously generate the highly ordered fibrils and fibers that in some tissues can be several feet long.

## The Two Subtypes: The Fibrillar and Non-Fibrillar Collagens

The fibril-forming or fibrillar collagens contain long and uninterrupted (Gly-XY-)$_n$ sequences (Figure 1A). They are among the most abundant proteins in nature,

 © 2004, Elsevier Inc. All Rights Reserved. 

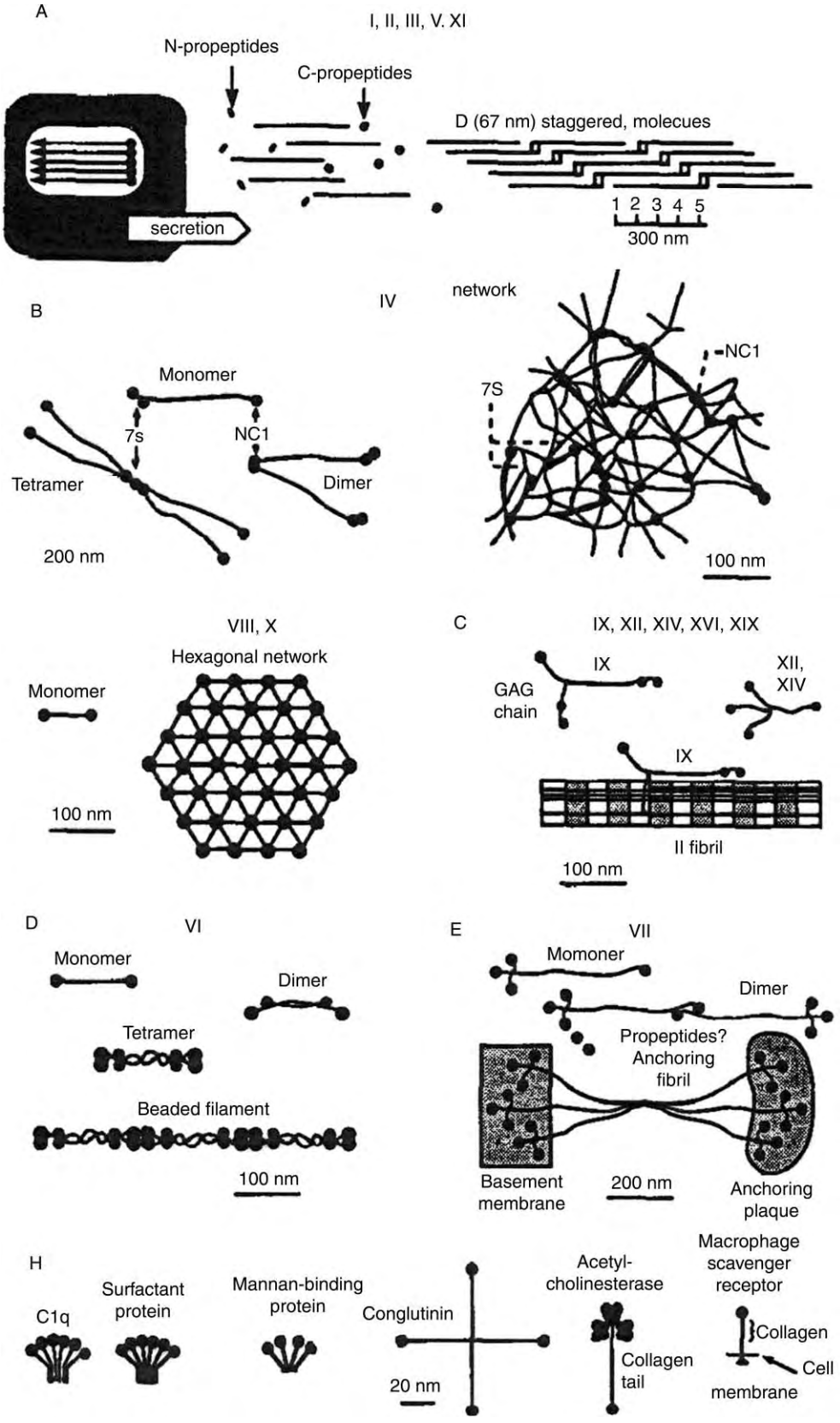



**FIGURE 1** Schematic for the structure of various collagens. Reproduced from Prockop, D. J., and Kivirikko, K. I. (1995). Collagens: Molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* **64**, 403–434.

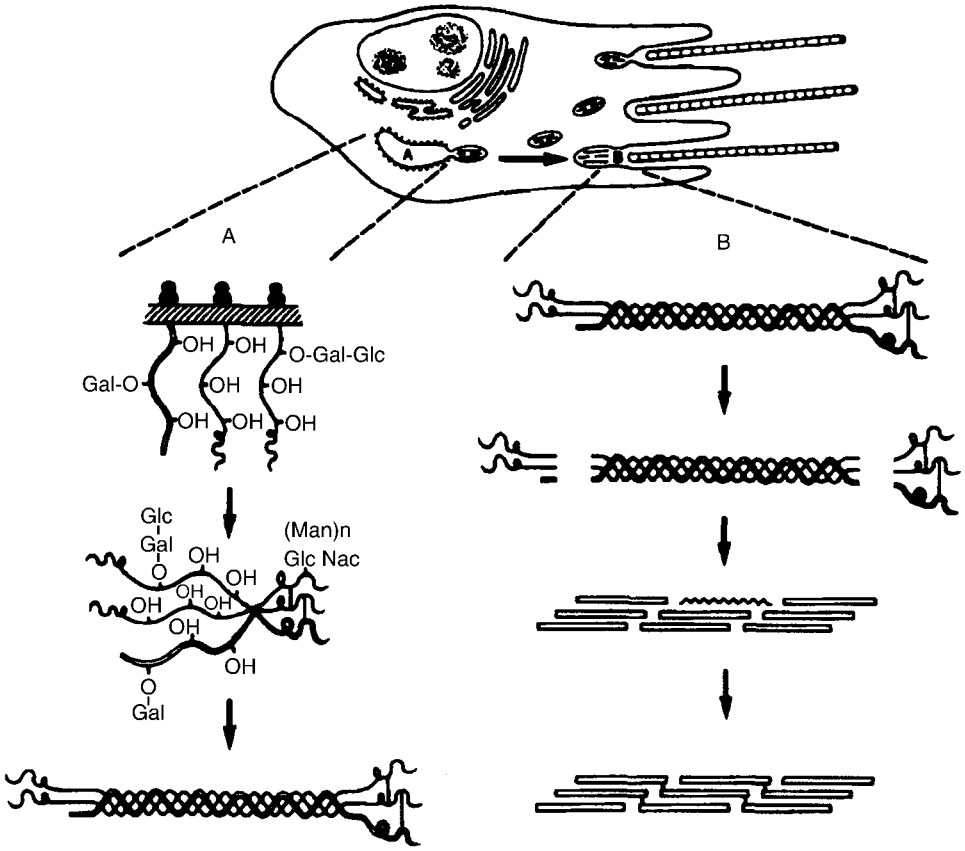



**FIGURE 2** Schematic for the biosynthesis of a fibril-forming collagen. (A) Intracellular events that involve posttranslational hydroxylations and glycosylations, association of polypeptide chains, and folding of the triple helix. (B) Extracellular events that involve cleavage of the N- and C-propeptides, self-assembly of collagen into fibrils, and cross-linking of the fibrils. Reproduced from Prockop, D. J., and Kivirikko, K. I. (1995). Collagens: Molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* **64**, 403–434.

and the most abundant of the fibrillar collagens are referred to as types I, II, and III. They are similar in structure in that each contains three polypeptide chains ($\alpha$-chains) with approximately 330 -Gly-XY- triplets. They are also similar in that the $\alpha$-chains spontaneously self-assemble into similar triple-helical monomers and the monomers spontaneously assemble into fibrils. Type I collagen accounts for well over 50% of the dry weight of ligaments, tendons, and demineralized bone. However, the biological properties of the fibrils depend on the still-undefined forces that orient the fibers in tissues. For example, the type I collagen fibrils in ligaments and tendons are present in parallel bundles of thick fibers. In skin, they are randomly oriented in the plane of the skin. In many regions of bone, type I collagen fibrils form highly symmetrical concentric helical arrays around the Haversian canal through which capillaries pass. Type II collagen is very similar to type I collagen in structure, but it is found almost exclusively in cartilage, where it forms very thin fibrils that trap highly charged proteoglycans and water. The result is an arcade-like structure that makes cartilage highly resilient to compression. Type III collagen is also similar to type I collagen in structure and its spontaneous assembly into fibrils. Type III collagen is associated in relatively small amounts with type I collagen in many tissues, but, for reasons that are not apparent, it is particularly abundant in the aorta.

The family of collagen proteins also includes a large number of proteins referred to as nonfibrillar collagens because either they contain only short $(\text{Gly-XY-})_n$ sequences or they contain $(\text{Gly-XY-})_n$ sequences that are interrupted by non-collagen sequences. As a result, the proteins do not assemble into highly ordered fibrils (Figure 1B–1H). One important example of a nonfibrillar collagen is the type IV collagen, which is an abundant constituent of basement membranes (Figure 1B). The protein is a large trimeric monomer with multiple $(\text{Gly-XY-})_n$ sequences that are interrupted by non-collagen sequences; it also contains large globular domains at both ends. The interruptions make the protein flexible, and the globular ends enable the protein to form flexible networks that

bind multiple other proteins. The flexible networks bind epithelial cells and form the barriers that protect the organism from the external environment. Other nonfibrillar collagens are much less abundant and fulfill more specialized functions. Some bind to the surfaces of fibers formed by fibrillar collagens and help either to regulate the assembly process or to provide binding sites for cells or other components of the extracellular matrix. For example, type IX is a short collagen that is flexible because its $(Gly\text{-}XY\text{-})_n$ sequences are interrupted by two non-collagenous sequences (Figure 1C). Type IX collagen binds to both fibrils of type II collagen and proteoglycans to provide one of the connecting links in the arcade-like structure of cartilage. Some nonfibrillar collagens contain very short $(Gly\text{-}XY\text{-})_n$ sequences and triple-helical domains that serve primarily as extension rods for more interactive domains on the ends of the same proteins (Figure 1H).

Collagens and collagen-like proteins are abundant in many multicellular organisms. For example, they are very abundant in diverse invertebrates such as sponges and worms, including the worms found in deep-sea hydrothermal vents. The smallest collagens appear to be minicollagens that contain only 12 to 16 -Gly-XY- repeats, and that are found in the nematocysts with which hydra sting their prey.

## The Need to Control the Self-Assembly of $(Gly\text{-}XY\text{-})_n$ Sequences

Viewed with evolution in mind, the special properties of $(Gly\text{-}XY\text{-})_n$ sequences can be seen to have a variety of different purposes. One of the most important of these purposes was to produce tough fibrils to hold together cells and thereby provide a means of developing multicellular organisms. However, it is clear that there had to be controls on the tendency of long polypeptides with repetitive $(Gly\text{-}XY\text{-})_n$ sequences to spontaneously assemble into large fibers. In living systems, the assembly of fibrils cannot be allowed to occur prematurely. If they did, the fibrils would either destroy the cell synthesizing the protein or encase the cell in an impenetrable matrix. Also, the fibrils must be strong but not as rigid as crystals. Their flexibility is critical for most tissues. It is apparently for these reasons that the synthesis of collagens is an elaborate process in which a series of specialized enzymes modifies the polypeptide chains as they are assembled on ribosomes and then pass into the cisternae of the endoplasmic reticulum en route to the Golgi apparatus and secretion from the cell.

## Why the Hydroxyproline?

One of the initial intriguing questions about collagens was why they contain large amounts of hydroxyproline, an amino acid very rarely found in other proteins. Early isotope studies demonstrated that after administration of labeled proline to rodents, both labeled proline and hydroxyproline were present in proteins isolated from the animals. After administration of labeled hydroxyproline, however, essentially no labeled hydroxyproline was recovered. Therefore, long before the genetic code was solved, it was apparent that the hydroxyproline in collagen was introduced by an event that did not occur during the initial assembly of the protein but that occurred as a posttranslational event. These observations led to the discovery that the hydroxyproline in collagens was synthesized by prolyl hydroxylase, an enzyme that specifically hydroxylates proline in the Y position in peptides with $(Gly\text{-}XY\text{-})_n$ sequences. The question of why hydroxyproline is introduced in this manner was resolved by demonstrating that if prolyl hydroxylase is inhibited, cells synthesize complete collagen polypeptide chains that cannot fold into a collagen triple helix unless the protein is cooled to about 15 °C below body temperature (Figure 2). The protein folds into a triple helix at body temperature only if about 100 proline residues in each of the 1000-amino-acid-long $\alpha$-chains of fibrillar collagen are hydroxylated to hydroxyproline. After the protein folds into the triple-helical conformation, prolyl hydroxylase can no longer modify it. In effect, prolyl hydroxylase titers the hydroxyproline content of the proteins so that it folds at body temperature. And, remarkably, the protein is not secreted from cells in significant amounts until it acquires the requisite content of hydroxyproline and folds into a triple helix. This elaborate system is one means of preventing the protein from folding too early in the biosynthetic process. The process by which prolyl hydroxylase gradually titers the hydroxyproline content of collagens serves a second function of ensuring that collagen fibrils are flexible. The monomers are secreted by cells before all the Y position proline residues are fully hydroxylated and before the molecule is a completely rigid rod. Instead, regions of the protein that have low contents of proline and hydroxyproline tend to "breathe" in the sense that they rapidly fold and unfold. Because the monomers are not rigid triple helices, the fibrils they assemble into are not fully crystalline and remain flexible. As might be expected, monomers for fibrillar collagens from organisms with body temperatures ranging from 18° to 40 °C completely unfold at about 4 °C above the body temperatures of the organisms. The fibrillar collagens from organisms with varying body temperatures

have different amino acids found in the X and Y positions that in part explain their different stabilities, but each begins as an unfolded protein that folds as its hydroxyproline content is titered to a critical level by prolyl hydroxylase. After the monomers assemble into fibrils, the lateral interactions of protein make the triple helix stable to about 16 °C above body temperature.

## Other Levels of Control

The process of fibril assembly by fibrillar collagens is also controlled at another step. The monomers are first synthesized as larger precursors that are known as procollagens. The procollagens have large non-collagenous domains at both ends that keep the monomers soluble and prevent them from self-assembling until the domains are cleaved by proteinases found outside the cells (Figure 2). Therefore, the proteinases help to regulate the formation of fibrils. In addition, the first fibrils formed begin to accumulate other proteins and components of the extracellular matrix on their surfaces that prevent fusion of the fibrils into large fibers. The rate at which these components are removed provides still another control on the formation of fibrils and fibers.

## One Surprise: The Enzymes Involved in Collagen Synthesis Have Other Functions

The biosynthesis of collagen involves processing by a series of separate enzymes that have a number of unusual properties (Figure 2). Because the enzymes carried out processing steps not seen in the synthesis of other proteins, it was initially felt that the enzymes were uniquely dedicated to production of collagen. Subsequently, it became apparent that several of the enzymes had important additional functions. For example, prolyl hydroxylase is unusual among the small number of enzymes that modify proteins in that it requires unfolded peptide substrates. It is also unusual in that it specifically hydroxylates prolyl residues in the Y position of -Gly-X-Pro- sequences. In addition, it is unusual in that the reaction uses iron, molecular oxygen, and ascorbate as well as $\alpha$-ketoglutarate that is oxidatively decarboxylated to succinate in the reaction. It was surprising, therefore, that cloning of the genes for the two subunits of the enzyme revealed that the $\beta$-subunit is identical to protein disulfide isomerase, an enzyme that is required to ensure the correct formation of disulfide bonds during the synthesis of a large number of non-collagen proteins. Perhaps even more surprising was the discovery of three similar but different prolyl hydroxylases that hydroxylate a single proline residue in hypoxia induction factor, a protein that triggers the response of tissues to ischemia. The proline residue in hypoxia induction factor must be hydroxylated for the protein to be degraded by proteosomes. One consequence of this discovery is that inhibitors of prolyl hydroxylase initially designed to limit the excessive deposits of collagen found in scars also increase tissue levels of hypoxia induction factor, thereby enhancing their response to hypoxia. Inhibitors of prolyl hydroxylase therefore appear to provide a new strategy for treatment of diseases such as anemias that are now treated with erythropoietin.

Similar surprises have been encountered with the proteinases that cleave the amino acid extensions on the ends of the procollagen precursors of fibrillar collagens and that keep the proteins from prematurely assembling into collagen fibers. The proteinase that cleaved the large C-terminal globular domains was a large zinc metalloproteinase with an unusual specificity for the similar but different sequences in the cleavage sites for the precursors of types I, II, and III collagens. Therefore, it seemed reasonable to assume that it was a protein with a unique function. Subsequent work demonstrated that the same enzyme was essential for processing a component of basement membranes (laminin-5) and for processing the enzyme (lysyl oxidase) that generates the aldehydes that form the covalent cross-links in fibrils of both collagen and elastin. Even more surprising was the discovery that isoforms of the same enzyme are essential in completely unrelated processes in early embryonic life: they establish a gradient for dorsolateral development by cleaving inhibitors of morphogenic proteins (decapentaplegic/BMP-2/BMP-4).

## Evolutionary Origins and Selective Pressures on (Gly-XY-)$_n$ Sequences

The manner in which (Gly-XY-)$_n$ sequences arose and were distributed across biology during evolution cannot be defined any more accurately than many other aspects of evolution. One clue seemed to come from the observation that the genes for the major fibrillar collagens have an unusual structure: most of the codons for the triple helix were found in short exons that were 54 bases long and coded for 18 triplets of (Gly-XY-)$_n$ sequences. Therefore, it appeared that evolutionary pressures had trapped the 54-base exon and replicated it to produce genes for many different collagens. Unfortunately, this attractive hypothesis was not substantiated by data on other collagen genes in which the exons are also relatively short, but the 54-base motif is not a common theme. Independent evidence on the selective pressures on the structure of collagens and the biosynthetic pathway is provided by data on mutations found in patients and a

few animals with genetic diseases. Studies on mutations in fibrillar collagens confirmed that the presence of glycine in (Gly-XY-)$_n$ sequences is critical. Hundreds of mutations that substitute an amino acid with a bulkier side chain for a single glycine distort the triple helix or prevent it from folding. As a result, the mutations produce a large series of diseases of bone, cartilage, blood vessels, and other tissues. Most of the mutations produce severe and rare diseases in children; a few produce variants of common diseases such as osteoporosis or osteoarthritis. The selective pressure for maintaining the signature acid sequences explains why the mutations in collagens differ only slightly from those in organisms that have evolved separately for millions of years. Mutations in the proteinase that cleaves the N-terminal extension on two fibrillar procollagens (types I and II) also produce severe diseases of the skeleton, skin, and other tissues. However, no mutations have been found in either prolyl hydroxylase or the proteinase that cleaves the C-terminal extensions on the procollagen precursors of fibrillar collagens. Such mutations are presumably lethal early in embryonic life.

## Summary

The impressive ability of (Gly-XY-)$_n$ sequences to spontaneously undergo self-assembly has been used time and again in biology to provide structures with a variety of important features. However, biological systems need safeguards to control the spontaneous self-assembly of the (Gly-XY-)$_n$ sequences. The principal safeguard is a biosynthetic pathway in which the protein is assembled through a series of complex steps that prevent premature self-assembly and modulate how perfectly the protein crystallizes.

## See Also the Following Articles

Amino Acid Metabolism • Collagenases • Metalloproteinases, Matrix

## Glossary

**collagen fibrils** Thin structures, 40 to 500 nm in diameter. Fibrils are assembled laterally into much larger fibers in tissues such as tendons.

**collagen monomer** A protein containing a collagen triple helix.

**collagen triple helix** A unique protein structure in which each of three chains with a (Gly-XY-)$_n$ sequence is coiled in a left-handed helix and the three chains are wrapped around each other in a more extended right-handed helix.

**procollagens** The soluble precursors of fibrillar collagens that contain large, non-collagenous extensions of both ends of the long triple-helical domains.

## Further Reading

Boudko, S., Frank, S., Kammerer, R. A., Stetefeld, J., Schulthess, T., Landwehr, R., Lustig, A., Bachinger, H. P., and Engel, J. (2002). Nucleation and propagation of the collagen triple helix in single-chain and trimerized peptides: Transition from third to first order kinetics. *J. Mol. Biol.* **317**, 459–470.

Canty, E. G., and Kadler, K. E. (2002). Collagen fibril biosynthesis in tendon: A review and recent insights. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **133**, 979–985.

Engel, J. (1997). Versatile collagens in invertebrates. *Science* **277**, 1785–1786.

Myllyharju, J., and Kivirikko, K. I. (2004). Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* **20**, 43–45.

Prockop, D. J., and Kivirikko, K. I. (1995). Collagens: Molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* **64**, 403–434.

Pugh, C. W., and Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: Role of the HIF system. *Nature Med.* **9**, 677–684.

## Biography

Darwin J. Prockop is Director of the Center for Gene Therapy at the Tulane University Health Sciences Center. His degrees include an A.B. from Haverford College; an Honors B.A. from Oxford University; an M.D. from the University of Pennsylvania; and a Ph.D. from George Washington University. His former research interests included collagen biosynthesis, structure and function of collagen genes, and genetic mutations that cause bone and cartilage diseases. His current interests are in defining the biological features of adult stem cells and using adult stem cells for therapy of genetic diseases involving collagen and diseases of the central nervous system, lung, and heart.

# Cyclic AMP Receptors of *Dictyostelium*

**Dale Hereld**
*The University of Texas Health Science Center, Houston, Texas, USA*

**Peter N. Devreotes**
*The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA*

**When confronted with starvation, the social amoeba *Dictyostelium discoideum* survives by undergoing multicellular development and sporulation. The coordination of these processes is achieved in part through intercellular communication using secreted adenosine 3′,5′-cyclic monophosphate (cAMP) and a family of cell-surface cAMP receptors (cARs). The cARs are examples of G protein-coupled receptors (GPCRs), which enable eukaryotic cells in general to sense and respond to a wide array of environmental and hormonal signals ranging from single photons to large glycoprotein hormones. Due to their involvement in diverse physiological processes, GPCR-targeted drugs are frequently employed in medicine to treat many common conditions including inflammation, hypertension, heart failure, and neurologic and psychiatric disorders. Because GPCRs and the pathways they regulate are conserved in virtually all eukaryotes examined to date, genetically tractable microbes such as *Dictyostelium* have contributed significantly to our understanding of GPCR function and regulation.**

## *Dictyostelium* Development and cAMP Signaling

*Dictyostelium discoideum* is an amoeba found in soil where it feeds on bacteria. In order to survive periods of starvation, $10^4$–$10^5$ amoebae aggregate and execute a 24 h developmental program that yields a fruiting body comprised of a round mass of spores held aloft by a slender stalk (Figure 1A). When nutrients return to the environment, spores germinate to yield amoebae which resume cell division.

Not long after Sutherland and his colleagues discovered cAMP to be an important intracellular second messenger in hormonal signaling, Konijn and his associates demonstrated that cAMP is a potent chemoattractant for *Dictyostelium* and correctly speculated that it was the so-called acrasin secreted by starving amoebae, which mediates their aggregation. Shaffer had proposed that the acrasin would be emitted periodically by cells at aggregation centers and relayed outwardly as waves by surrounding cells. Indeed, exogenous cAMP was shown to elicit the transient activation of adenylyl cyclase and secretion of cAMP. Tomchik and Devreotes later demonstrated the concentric waves of extracellular cAMP waves which arise every ~6 min at aggregation centers and travel radially outward through aggregating populations. Subsequent pharmacologic characterization of these responses established the framework for the identification of the cAMP receptors.

## Identification and Properties of cAMP Receptors

The molecular identification of the first cAMP receptor (cAR1) began with its photoaffinity labeling with the cAMP analogue, 8-azido-[$^{32}$P] cAMP. This approach identified a protein of either 40 or 43 kDa depending on whether or not the cells had been exposed to cAMP, suggesting the existence of a reversible, ligand-induced modification which proved to be phosphorylation. Radiolabeling of cells with [$^{32}$P] phosphate permitted purification of the 43 kDa phosphorylated form of the receptor. Antibodies directed against purified cAR1 lead to the isolation of a cDNA that encoded a protein of the expected size and hybridized to an mRNA, expressed transiently in early development, consistent with cAMP binding. Formal proof that the isolated cDNA did indeed encode cAR1 came from expression of the cDNA, which resulted in increased cAMP binding, and disruption of the corresponding gene, which obviated cAMP binding and cAR1-mediated responses. The deduced sequence of cAR1 possessed seven putative transmembrane domains (Figure 2) and exhibited weak homology to mammalian G protein-coupled receptors (GPCRs). cAMP stimulation of GTP binding to isolated membranes and GTP hydrolysis was further evidence

 © 2004, Elsevier Inc. All Rights Reserved. 

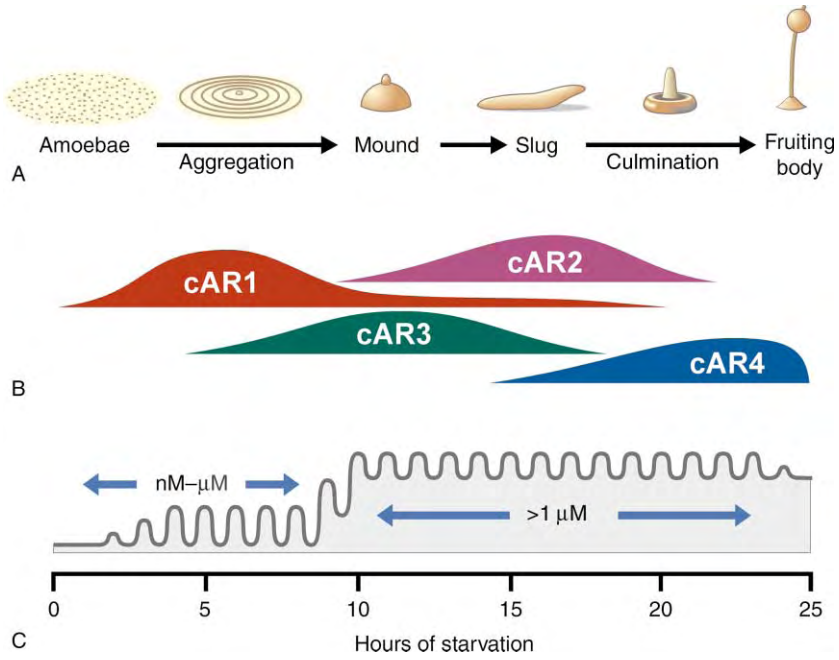



**FIGURE 1** Correlation of: (A) developmental morphology, (B) cAR expression, and (C) extracellular cAMP levels. See text for additional details.

that cAR1 is indeed coupled to a G protein. This together with studies of yeast pheromone signaling provided the earliest indications that virtually all eukaryotes have inherited these ancient sensory mechanisms.

Three other highly homologous cAMP receptors, designated cAR2–4, were subsequently identified by hybridization with a cAR1 probe. The cARs are expressed successively during development, peaking in expression at roughly 5 h intervals in the order: cAR1,

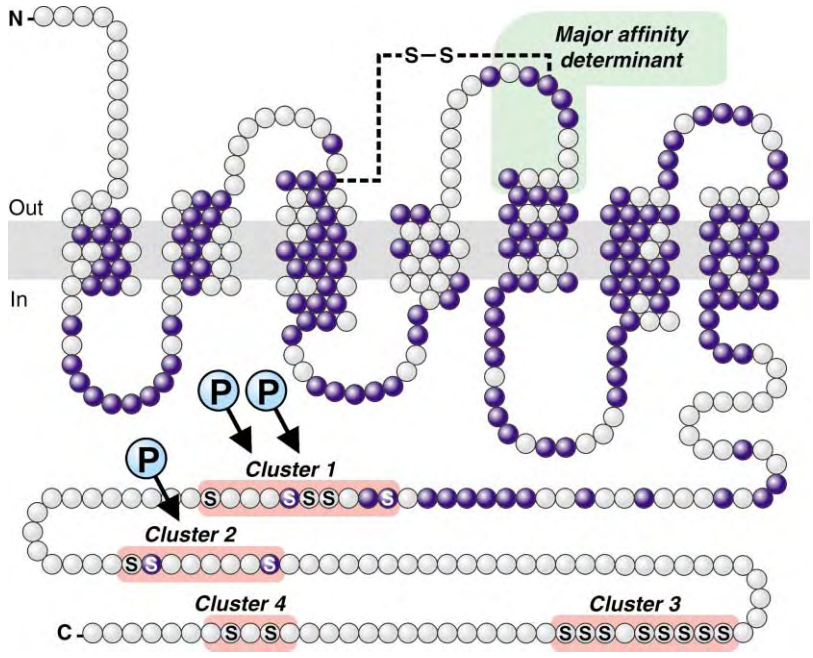



**FIGURE 2** Model of cAR1. Dark blue spheres represent amino acids conserved in all four cARs. Densely packed amino acids in the plane of the membrane (gray bar) signify the seven-hydrophobic-transmembrane helices. Nonconserved amino acids (light gray spheres) in the region labeled major affinity determinant are largely responsible for the widely differing cAMP affinities of cAR1 and cAR2 (and possibly other cARs). Clusters of serines in the C-terminal cytoplasmic domain (S), the distribution of ligand-induced phosphorylation (P), and the putative disulfide bridge (––S–S––) are also indicated. cAR2–4 differ in the lengths and sequences of their C-terminal cytoplasmic domains.

cAR3, cAR2, cAR4 (Figure 1B). Disruption of the genes encoding each cAR results in developmental defects consistent with the timing of their expression. cAR1$^-$ cells fail to aggregate. cAR2$^-$ cells arrest shortly after aggregating at the mound stage. cAR3 gene disruption has been variously reported either to have no apparent effect or to interfere with spore cell differentiation late in the mound stage and consequently yield fruiting bodies predominantly comprised of stalk cells. cAR4$^-$ cells develop normally beyond the mound stage but exhibit defects in culmination, resulting in mis-shapen fruiting bodies.

The cARs differ markedly in their affinities for cAMP. The early cARs, cAR1 and cAR3, have high affinities (i.e., low- to mid-nM $K_d$'s), whereas those expressed later in development, cAR2 and cAR4, have low affinities ($K_d$'s $> 1$ μM). These affinities are appropriate for the extracellular cAMP concentrations that exist at these stages of development (Figure 1C). During aggregation, the cAMP signal oscillates from sub-nM to near-μM concentrations. In contrast, external cAMP oscillates at elevated concentrations exceeding 1 μM in the multicellular stages. Nonconserved residues in the second extracellular loop largely determine whether a cAR has a high or low affinity (Figure 2). By analogy with rhodopsin, this extracellular loop, positioned by disulfide linkage to the extracellular end of the third transmembrane helix, is likely to lie at the entrance to the cAMP binding cleft of cARs and, thereby, influence binding.

## Signaling Pathways

cAR1 is perhaps the most versatile GPCR yet to be characterized as it regulates a wide range of downstream effectors and biological responses. Consequently, it serves as a valuable model for understanding diverse modes of GPCR signaling. cAR1 mediates three principal cellular responses during aggregation: (1) propagation of cAMP waves, (2) chemotaxis up the cAMP gradient of each oncoming wave, and (3) regulation of genes required for development. The pathways underlying these responses have been determined to a large extent (Figure 3A). Most striking is the dichotomy between signaling pathways that involve G proteins and those that do not. Comparatively less is known about the pathways governed by cAR2–4 in large measure due to technical challenges posed by multicellularity, although significant progress has been made towards elucidating mechanisms by which these cARs promote cell differentiation in multicellular stages.

### G Protein-Dependent Pathways

*Dictyostelium* possesses at least nine heterotrimeric G proteins composed of distinct $\alpha$-subunits (designated G$\alpha$1–9) and common $\beta$- and $\gamma$-subunits. Genetic and biochemical evidence indicates that G$\alpha2\beta\gamma$ is the principal G protein to which cAR1 couples. Activation of G$\alpha2\beta\gamma$ by cAR1 liberates the G$\beta\gamma$ dimer and GTP-bound G$\alpha$2, which in turn activate various effectors.

The G$\beta\gamma$ dimer is believed to activate the aggregation-stage adenylyl cyclase (ACA) by a mechanism involving activation of phosphoinositide-3-kinase (PI3K), which converts the membrane phospholipid phosphatidylinositol-4,5-bisphosphate ($PIP_2$) into phosphatidylinositol-3,4,5-trisphosphate ($PIP_3$). $PIP_3$, in turn, binds to the PH domain of cytosolic regulator of adenylyl cyclase (CRAC), thus recruiting it to the plasma membrane. How CRAC then activates ACA remains to be determined. The resulting cAMP

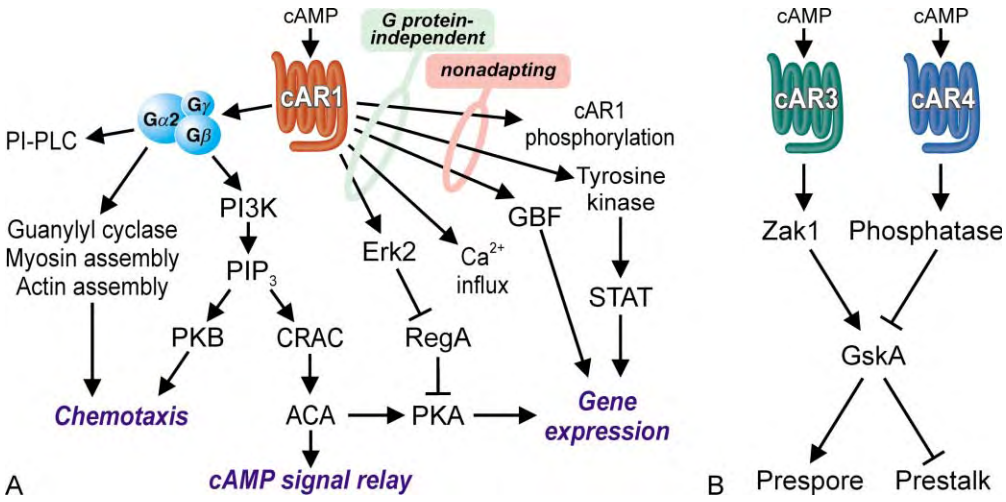



**FIGURE 3** cAMP receptor signaling pathways. Pointed (↓) and flat-headed (⊥) arrows indicate activation and inhibition, respectively. (A) cAR1 pathways leading to chemotaxis, cAMP signal relay, and gene regulation. G protein-independent and nonadapting pathways are indicated. In multicellular stages, cAR2–4 might activate some of the pathways shown for cAR1. (B) Role of cARs in the GskA-mediated determination of cell fate. cAR2 (not shown) might promote prestalk cell differentiation at the mound stage in the manner shown for cAR4.

functions intracellularly via protein kinase A (PKA) to modulate gene expression and, in addition, is secreted from the cell in order to relay the cAMP signal to neighboring cells.

G$\beta\gamma$-mediated increases in $PIP_3$ levels also recruit other PH domain-containing proteins to the plasma membrane including protein kinase B (PKB or Akt), which has been shown to be critical for chemotaxis. In cells undergoing chemotaxis in a shallow cAMP gradient, PKB and other PH domain-containing proteins localize exclusively at the cells' leading edge where they presumably promote actin assembly and pseudopod extension. These mechanistic insights into cAR1-mediated chemotaxis, namely the involvement of PI3K activation and recruitment of PKB to the leading edge, have since been found to also pertain to the chemotaxis of a variety of mammalian cells including neutrophils.

The G$\alpha$2 subunit is implicated in the cAR1-dependent activation of guanylyl cyclase (cGMP) which provides another important input to the chemotaxis machinery. The product of cGMP regulates myosin heavy chain kinases and, thereby, promotes the assembly of conventional myosin II in posterior and lateral regions of chemotaxing cells where it can propel the rear of the cell forward upon contraction and suppress lateral pseudopod formation.

## G Protein-Independent Pathways

Several cAR-mediated responses appear to be G protein-independent based on their preservation in cells lacking what is believed to be the sole G protein $\beta$-subunit gene. These include phosphorylation of cAR1, uptake of $Ca^{2+}$, and activation of the mitogen-activated protein kinase Erk2, the transcriptional regulators GBF and STAT, and the GSK3 homologue GskA.

cAR1 is phosphorylated on serine residues within its C-terminal cytoplasmic domain. The 18 serines in this domain exist in four clusters (Figure 2). In unstimulated cells, cAR1 is basally phosphorylated within clusters 2 and 3. Upon stimulation with cAMP, cAR1 becomes reversibly hyperphosphorylated due to the addition of approximately two phosphates within cluster 1 and a third phosphate within cluster 2. Cluster 1 phosphorylation causes the 40–43 kDa electrophoretic shift. Other cARs also undergo cAMP-induced phosphorylation commensurate with their affinities.

Erk2 promotes the intracellular accumulation of cAMP by negatively regulating RegA, a cAMP-specific phosphodiesterase. Erk2 is presumably the third of three kinases in a typical MAP kinase cascade. The identity of the upstream kinases as well as the G protein-independent mechanism by which cAR1 activates the cascade remain to be determined.

cAMP triggers the rapid and transient influx of $Ca^{2+}$ ions in aggregation-competent cells. This is mediated largely by cAR1 due to its natural abundance at this stage in development. However, expression of cARs in vegetative cells indicates that the components required for uptake are expressed at this stage and that other cARs can also mediate $Ca^{2+}$ uptake. The magnitude of $Ca^{2+}$ uptake is roughly proportional to receptor level, indicating that cARs are the limiting components for this response.

G-box binding factor (GBF) binds G-rich elements in early postaggregative genes and is required for their induction by cAMP. Gene disruptions suggest that either cAR1 or cAR3 mediate the G protein-independent activation of GBF. In contrast to many aggregation-stage genes whose expression requires periodic cAMP pulses that mimic natural cAMP waves, GBF-mediated gene expression is induced by constant cAMP, indicating that it is not subject to adaptation. Thus, the GBF pathway is appropriately activated upon aggregation when extracellular cAMP rises to levels that persistently occupy cAR1.

STAT proteins have been extensively studied in the context of cytokine signaling in mammalian immune cells. In *Dictyostelium*, exogenous cAMP triggers STATa's phosphorylation on tyrosine, SH2 domain-mediated dimerization, and translocation to the nucleus where it governs prestalk gene expression. cAR1 is required for this response but it can be substituted in this capacity with cAR2, suggesting that multiple cARs might activate STATa during development. The cAR-activated tyrosine kinase involved remains to be identified.

GskA, a homologue of glycogen synthase kinase-3 (GSK3), is an important regulator of cell fate in *Dictyostelium* and cARs play key roles in regulating its activity. GskA activity promotes spore cell differentiation, whereas inactivity results in stalk cell differentiation (Figure 3B). Independent of G proteins, cAR3 activates the nonreceptor tyrosine kinase Zak1 which, in turn, phosphorylates and activates GskA. On the other hand, cAR4 (and perhaps cAR2) activates a phosphotyrosine phosphatase, resulting in the dephosphorylation and inactivation of GskA. The role of G proteins in the latter process, if any, is not known. Therefore, cAR3 signaling promotes spore differentiation and cAR2 and cAR4 favor stalk cell differentiation. Because cAR2 and cAR4 are expressed predominantly in prestalk cells, it is unclear whether these mechanisms determine cell fate or act thereafter in cell-type maintenance.

One known target of cAMP-activated GskA is STATa. GskA phosphorylates multiple serines of STATa, causing it to be exported from the nucleus. This is one mechanism by which cAR3 signaling might oppose prestalk differentiation. As has been described for other developmental systems, GskA may also determine cell fate by phosphorylating $\beta$-catenin and thus targeting this transcriptional coactivator for destruction.

### Mechanisms of G Protein-Independent Signaling

It remains to be determined how cAMP-occupied cAR1 communicates with and activates these effectors. By analogy with G proteins and also mammalian G protein-coupled receptor kinases (GRKs), the yet to be identified cAR kinase might interact selectively with the ligand-occupied conformation of the receptor's cytoplasmic loops. Other G protein-independent processes such as $Ca^{2+}$ uptake could involve lateral signal transduction within the plane of the membrane by direct interaction of cAR1 with another integral membrane protein, analogous to the interaction of *Halobacterium* sensory rhodopsins (SI and SRII) with their associated histidine kinases (HtrI and HtrII).

## Desensitization Mechanisms

In general, receptor-mediated responses are governed by various desensitization mechanisms which attenuate the cell's responsiveness. cAMP triggers the sequential phosphorylation ($t_{1/2} \sim 2$ min) and internalization ($t_{1/2} \sim 15$ min) of cAR1. cAR1 phosphorylation causes a several-fold reduction in the receptor's intrinsic affinity for cAMP, which may extend the range of cAMP concentrations to which the receptor can respond during aggregation. In addition, preliminary results indicate that phosphorylation of cAR1 is a prerequisite for its internalization as in other systems. Prolonged cAMP exposure results in down-regulation of cAR1 levels, the combined effect of diminished cAR1 gene transcription, and cAR1 degradation. Degradation presumably results from delivery of internalized receptors to lysosomes.

On a more rapid timescale, nearly all of the cAR1-mediated responses to abrupt cAMP increases are transient, returning to prestimulus levels in 30 s to several minutes despite constant stimulation. This rapid and reversible attenuation of responses is referred to as adaptation. The few responses that do not adapt include cAR1 phosphorylation and GBF activation. The mechanisms of adaptation are poorly understood and might be distinct for each pathway. cAR1 phosphorylation appears not to be involved as elimination of phosphorylated serine residues in cAR1 by site-directed mutagenesis has little impact on the kinetics of these responses.

Cells adapted to one cAMP concentration can respond to yet higher concentrations (provided the receptor is not saturated). This observation indicates that adaptation is a graded signal that is just sufficient to offset the excitatory signal, the strength of which also reflects receptor occupancy. For the adenylyl cyclase pathway, adaptation can be explained by the transient translocation of PI3K to the plasma membrane and the subsequent degradation of $PIP_3$ by the phosphatase PTEN. In addition, FRET experiments indicate that the G protein is persistently dissociated in adapted cells. Taken together, these findings suggest that an adaptation pathway emanates from cAR1 and acts upon the adenylyl cyclase excitatory pathway somewhere beyond the G protein, causing PI3K to be released from the membrane. Because PKB activation also depends on PI3K, the same adaptation mechanism is likely to govern chemotaxis.

## Gradient Sensing in Chemotaxis

Temporal challenges with fixed cAMP concentrations as described above have been invaluable for deciphering cAR-mediated pathways and revealing the existence of adaptation mechanisms. However, natural cAMP waves also contain spatial information which the cells must rapidly and accurately sense for efficient chemotaxis. Although cAR1 is uniformly distributed in the plasma membrane, shallow cAMP gradients differing by as little as 2% across the length of the cell prompt highly asymmetric localization of various proteins, indicating that the cell senses and amplifies small differences in cAR1 occupancy on this surface. Proteins with PH domains including PKB and CRAC are highly localized to the plasma membrane of cell's leading (or anterior) edge, indicative of elevated $PIP_3$ levels. This reflects the recruitment of PI3K to the anterior plasma membrane and its subsequent activation. The membrane translocation of PI3K is mediated by its N-terminal domain which presumably binds an entity in the plasma membrane that is generated in response to cAR1 activation. Conversely, PTEN is associated with the posterior plasma membrane via specific interactions with $PIP_2$. Thus, PTEN is excluded from the anterior membrane where PI3K actively converts $PIP_2$ to $PIP_3$ and instead localizes to posterior regions where $PIP_2$ should be more abundant. Localization of the antagonistic activities of PI3K and PTEN to opposing poles of the cell should result in a steep gradient of $PIP_3$ and associated PH domain-containing proteins. Precisely how the cell amplifies the directional information of a shallow chemoattractant gradient to achieve extreme gradients of activities within the cell is likely to be critically important for efficient chemotaxis and is under intensive investigation.

### See Also the Following Articles

Adenylyl Cyclases • G Protein-Coupled Receptor Kinases and Arrestins • Phosphatidylinositol Bisphosphate and Trisphosphate

## Glossary

**chemotaxis** Directed movement of cells toward (or away from) the source of diffusable chemoattractant (or repellant) molecules.

**G protein** Heterotrimeric proteins with inactive (GDP-bound) and active (GTP-bound) states which are activated by ligand-occupied receptors and, in turn, activate downstream targets within the cell.

**G protein-coupled receptors** Cell surface, integral membrane proteins possessing seven membrane-spanning $\alpha$-helices and usually capable of activating cytosolic G proteins upon binding specific extracellular signaling molecules (also known as seven-transmembrane or serpentine receptors).

**signal transduction** Molecular events by which the perception of an extracellular signal by a cell is translated into an appropriate cellular response.

## Further Reading

Brzostowski, J., and Kimmel, A. (2001). Signaling at zero G: G-protein-independent functions for 7-TM receptors. *Trends Biochem. Sci.* **26**, 291–297.

Chung, C. Y., Funamoto, S., and Firtel, R. A. (2001). Signaling pathways controlling cell polarity and chemotaxis. *Trends Biochem. Sci.* **26**, 557–566.

Iijima, M., Huang, Y. E., and Devreotes, P. (2002). Temporal and spatial regulation of chemotaxis. *Dev. Cell* **3**, 469–478.

Kessin, R. (2001). *Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity.* Cambridge University Press, Cambridge, UK.

Kim, L., and Kimmel, A. R. (2000). GSK3, a master switch regulating cell-fate specification and tumorigenesis. *Curr. Opin. Genet. Develop.* **10**, 508–514.

## Biography

Peter N. Devreotes is a Professor and Chair of the Department of Cell Biology at the Johns Hopkins University School of Medicine in Baltimore, Maryland. His principal research interests include the molecular mechanisms of cell-to-cell signaling, chemoattractant gradient sensing, and chemotaxis. He holds a Ph.D. from Johns Hopkins and obtained postdoctoral training at the University of Chicago.

Dale Hereld, who earned an M.D. and a Ph.D. from Johns Hopkins, was a Damon Runyon-Walter Winchell Fellow under Dr. Devreotes and presently is an Assistant Professor at the University of Texas Health Science Center in Houston, Texas.

# Cyclic GMP Phosphodiesterases

**Sharron H. Francis and Jackie D. Corbin**
*Vanderbilt University School of Medicine, Nashville, Tennessee, USA*

**Cyclic nucleotide (cN) phosphodiesterases (PDEs) are phosphohydrolases that specifically cleave the cyclic phosphodiester bond of 3′,5′-cyclic GMP (cGMP) or 3′,5′-cyclic AMP (cAMP) (Figure 1) to the respective 5′-nucleotides thereby inactivating these signaling molecules. PDEs are largely responsible for lowering the intracellular cN content, and the activities and concentrations of these enzymes are rigorously regulated. Some PDEs are highly specific for either cAMP or cGMP, but others, i.e., dual-specificity PDEs, hydrolyze both. Both types of PDEs contribute importantly to modulation of cGMP-signaling pathways. The competition of cGMP and cAMP for hydrolysis by the dual specificity PDEs can also alter the signaling in the respective pathways. PDEs are integral components in cN signaling pathways (Figure 2) from *Paramecia* through mammals, and in humans, these enzymes are the pharmacological targets for treatment of a number of medical problems including hypertension, male erectile dysfunction, depression, and asthma.**

**Cyclic GMP and cAMP are prominent second-messenger molecules (Figure 1) and are synthesized by guanylyl cyclases (GC) and adenylyl cyclases (AC), respectively. The balance between the activities of the cyclases and PDEs determines cellular cN levels (see cGMP-signaling pathway, Figure 2). The cyclases specifically synthesize either cAMP or cGMP. The cyclic phosphate ring of cGMP and cAMP is a key feature by which PDEs recognize their substrates, and it is highly resistant to most other phosphohydrolases. Nucleotides lacking this ring do not significantly interact with the catalytic sites of PDEs even at high concentrations. Structural features of the purine (Figure 1) provide the next most critical feature for interaction of the cN with PDEs. For cGMP-specific PDEs, interaction with the oxygen at the C6 position is extremely important.**

## Distribution of cAMP and cGMP

Cyclic AMP is apparently present in all eukaryotes, but cGMP is less widespread (Figure 1). The yeast genome lacks identifiable sequences for GCs or known cGMP-binding domains. However, *Dictyostelium, Paramecium, Tetrahymena, Trypanosomatids*, and *Plasmodium* contain GCs. The role of cGMP in most of these organisms is not understood, but in *Dictyostelium*, cGMP participates importantly in chemotaxis and osmoregulation. In mammals, cGMP-signaling pathways are prominent in many processes including smooth muscle relaxation, visual transduction, platelet function, neuronal viability, bone growth, apoptosis, water and electrolyte homeostasis, and aldosterone synthesis.

## Classification of PDEs

Three evolutionarily distinct classes of PDEs, class I, class II, and class III have been identified; to date, class III contains one PDE that hydrolyzes cAMP and will not be discussed further. Both class I and class II PDEs occur in some species, e.g., *Dictyostelium* and *Saccharomyces*. All known mammalian PDEs and a few PDEs in other species are class I PDEs. In eukaryotes, the cN-binding sites in PDEs are distinguished from those of other known intracellular cN receptors both functionally and evolutionarily; these include cN-binding proteins such as the cAMP- and cGMP-dependent protein kinases (PKA and PKG) which belong to the bacterial catabolite gene activator protein (CAP-related family), the cGMP-binding GAF domains in some PDEs, and some anion transporters.

## Class I PDEs

The class I PDE superfamily in mammals contains 11 PDE families that are products of separate genes. Classification is based on the extent of similarity in the DNA sequences. Three are cAMP-specific PDEs (PDEs 4, 7, and 8), three are "cGMP-specific" PDEs (PDEs 5, 6, and 9), and four are dual-specificity PDEs (PDEs 1, 2, 3, 10, and 11) (Figure 3). Among isoforms of the latter group, the relative affinities and turnover rates for cGMP and cAMP can vary significantly as exemplified by PDE1.

### Domain Structure of Class I PDEs

Each class I PDE monomer is a chimeric protein that includes a conserved catalytic domain (C domain) of ~270 amino acids that is located more C-terminal to its regulatory domain (R domain); 18 amino acids are

 © 2004, Elsevier Inc. All Rights Reserved. 

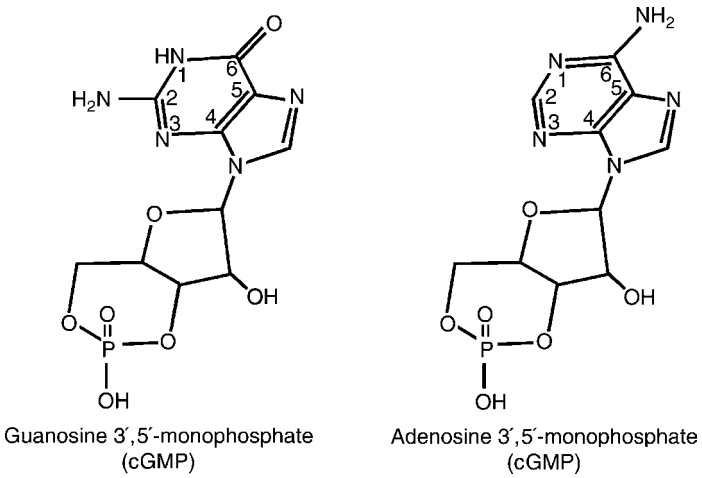



**FIGURE 1** Molecular structures of cGMP and cAMP. The structures of cGMP and cAMP differ only at the N-1, C-6, and C-2 positions, but these differences provide for the specific and selective interaction with PDE catalytic sites. The *syn* conformation of both nucleotides is shown.

invariant among mammalian class I PDEs. Among all known class I PEDs, only 14 amino acids are invariant. These most likely provide for critical structural and catalytic features. Diverse regulatory features for each family modulate enzyme functions (Figure 3). The R domains contain varied subdomains including phosphorylation sites, autoinhibitory sequences, subcellular localization signals, dimerization motifs, and GAFs; GAFs are ancient domains of ~120 amino acids that occur in five PDE families. The name is derived from recognition of this motif in a number of diverse proteins (cGMP-binding PDEs, *Anabaena a*denylyl cyclase, and *Escherichia coli F*hla transcription factor). Depending on the PDE in which they are located, GAFs can contribute to for dimerization, interaction with other proteins or with ligands such as cGMP; cAMP interacts weakly with these sites in PDE2, but it does not appear to bind to these sites in other PDEs.

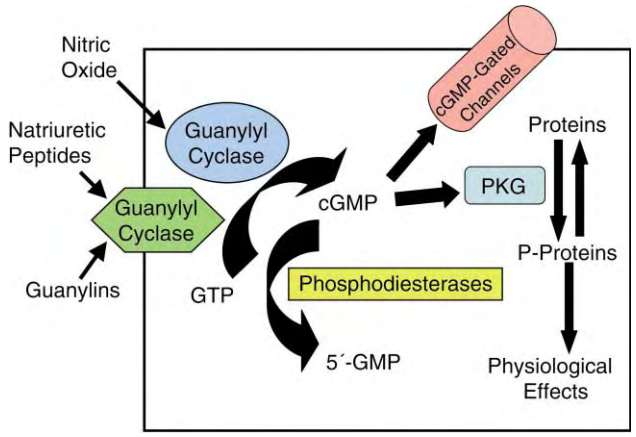



**FIGURE 2** Model of cGMP signaling in cells. Intracellular levels of cGMP are determined by the relative activities of guanylyl cyclases and phosphodiesterases. There are two families of guanylyl cyclases; the cytosolic form is activated by nitric oxide and a membrane-bound form is activated by natriuretic peptides and guanylins. Cyclic GMP breakdown is catalyzed by phosphodiesterases that are either cGMP-specific or PDEs that hydrolyzed both cGMP and cAMP, i.e., dual-specificity PDEs. Cyclic GMP interacts with the cGMP-dependent protein kinase (PKG), cGMP-gated cation channels, and cGMP-binding phosphodiesterases to bring about its physiological effects.

## C Domains of Class I PDEs

The C domains of class I PDEs contain two prominent functional subdomains; one provides for binding divalent cation(s) and another provides for interaction with the cN purine. Metal and cN apparently bind independently. The subdomains are closely juxtaposed in the tertiary structure in order to effect catalysis. Crystallographic structures of cAMP-specific PDEs (PDE4B and PDE4D), cGMP-specific PDE5, and site-directed mutagenesis of a number of PDEs have identified important amino acids in each subdomain.

### Metal Requirements of Class I PDEs

PDEs require divalent cation(s) to support catalysis. Evidence suggests that class I PDEs contain two metals that are bound in close proximity and are critical to the catalytic process. In two cGMP-specific PDEs (PDEs 5 and 6), $Zn^{2+}$ is required for catalysis, and PDE6 contains three $Zn^{2+}$ molecules. Results of site-directed mutagenesis and X-ray crystallographic structures of the catalytic domains of PDEs 4 and 5 indicate that these PDEs contain a tightly bound $Zn^{2+}$ which is coordinated by histidines, aspartic acid, and water molecules. The second metal is coordinated through interactions with an aspartic acid and several water molecules that are in turn linked to histidines and aspartic acid. The metal occupying the second

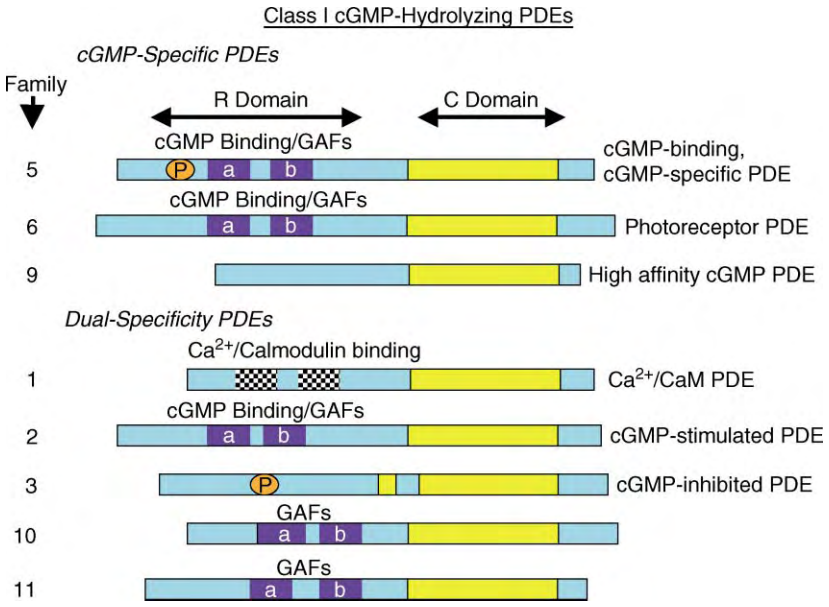



**FIGURE 3** Schematic depiction of structural features of mammalian class I cGMP-hydrolyzing PDEs. Arrangements of the regulatory (R) domains and catalytic (C) domains are shown for a monomer, although all known mammalians PDEs exist as dimers. Known sites of phosphorylation (P) are indicated. The cGMP binding/GAFs label denotes allosteric cGMP-binding that is associated with GAF domains in PDEs 2, 5, and 6; all isoforms of these families contain two GAF domains. The role of the GAF domains in PDEs 10 and 11 is not known; isoforms within the PDE11 family contain 1, 2, or no complete GAF domains. The open block in PDE3 is a novel 44 amino acid insert in the catalytic domain of this family. Cross-hatched boxes denote calmodulin-binding domains (1 or 2) in the R domain of PDE1

metal site in PDEs is still unclear and may vary. Both $Mg^{2+}$ and $Mn^{2+}$ have been suggested to be the physiologically relevant cations, but this remains to be definitively demonstrated. Nucleophilic attack by an activated hydroxyl from solvent water that bridges the metals is thought to cleave the phosphodiester bond.

### Features that Contribute to cN Specificity

The molecular basis that provides for the affinity and selectivity of PDEs for either cN is poorly understood. Substitution of two amino acids by point mutations shifts the cGMP/cAMP specificity of PDE5 ~100-fold. PDE11, a dual-specificity PDE, and PDE5, a cGMP-specific PDE, are most similar among PDEs. X-ray crystallographic structures of PDEs 4 and 5 suggest that the orientation of the side chain of an invariant glutamine is a critical determinant of specificity for interaction with the purine ring in either cGMP or cAMP.

In solution, cNs are in equilibrium between two conformations, *syn* and *anti*, based on orientation between the purine and ribose moieties. The preferred configuration varies among PDE families, but the *anti* conformer is most commonly preferred. Large substitutions on the ribose 2′-OH or on the N-1 and C-2 positions of the purine are well tolerated by the catalytic sites of both cGMP-specific PDEs 5 and 6 and the dual-specificity PDEs 1 and 2. Some of these analogues are hydrolyzed by the enzymes.

### Interaction of Inhibitors with Class I PDEs

Specific structural features in the catalytic sites of cGMP-PDEs provide for interaction with different inhibitors. Some inhibitors compete for access to the catalytic site by mimicking the structure of cGMP. This is true for two specific inhibitors of PDE5, i.e., sildenafil (Viagra) and vardenafil (Levitra); tadalafil (Cialis) also competes with cGMP for the catalytic site, but its structure is not closely patterned on that of cGMP, and it forms different contacts with PDE5 than occur with sildenafil or vardenafil. Studies based on site-directed mutagenesis indicate that sildenafil utilizes many of the amino acids that provide for interaction of cGMP with PDE5. Affinity of PDE5 for these inhibitors is ~1000–10 000 times greater than for cGMP, so additional contacts must be involved.

PDEs 5, 6, and 11 are closely related and share ~50% amino acid sequence homology. Sildenafil inhibits both PDEs 5 and 6, but it has ~4–10 fold lower affinity for isoforms of PDE6 than for PDE5; it is a very weak inhibitor of PDE11. In contrast tadalafil (Cialis) potently inhibits PDE5, is ~40-fold less effective against PDE11, and has little effect on PDE6. PDE1, a dual-specificity PDE, is inhibited by sildenafil with ~100-fold lower potency than that for PDE5. The catalytic sites of

PDEs 1, 5, and 6 have similar preferences for cN analogues, so it is likely that sildenafil exploits shared structural features among these PDEs. In contrast, some other inhibitors selectively inhibit the dual-specificity PDEs 1, 2, and 3 and are ineffective against PDEs 5 and 6. Future development of PDE inhibitors will undoubtedly map the patterns of molecular selectivity to identify compounds that preferentially inhibit particular cGMP PDEs. PDE6 isoforms are novel in that they are specifically inhibited by a small protein, P$\gamma$, that is abundant in photoreceptor cells. P$\gamma$ inhibitors appear to be entirely specific for PDE6 isoforms.

## Regulation of cGMP-Hydrolyzing Class I PDEs

Cyclic GMP-hydrolyzing PDEs are regulated by both short-term and long-term regulatory mechanisms. These include ligand activation (e.g., $Ca^{2+}$/calmodulin (PDE1) and cGMP binding to allosteric sites provided by one or more GAF domains (PDEs 2, 5, 6, and perhaps 10 and 11), phosphorylation (PDEs 1, 3, 5, and 11) (Figure 3), and increases in protein levels. In a physiological setting, these provide for sensitive control of PDE activities.

In PDEs 2, 5, and 6, GAFs provide for allosteric cGMP binding, and are evolutionarily distinct from the catalytic sites. Molecular determinants for cGMP interaction with the catalytic site differ markedly from those for cGMP binding to GAF sites. Cyclic GMP is the only ligand that has been shown to bind to GAFs in PDEs; this has been reported only for PDEs 2, 5, and 6. In PDE2, cAMP competes with cGMP binding but with $>10$-fold lower affinity; it does not measurably compete with cGMP binding in PDEs 5 and 6. In the X-ray crystallographic structure of the PDE2 R domain, cGMP is almost entirely buried within the GAF b site. The tight fit of cGMP agrees well with the fact that few cGMP analogues compete with cGMP at this site. In PDE5, allosteric cGMP binding occurs in the GAF a site.

## Characteristics of cGMP-Specific Class I PDEs (PDEs 5, 6, and 9)

### PDE5

PDE5 is known as the cGMP-binding, cGMP-specific PDE. There are four isoforms. PDE5 has $>100$-fold selectivity for cGMP over cAMP at both its catalytic site and allosteric sites; it is a homodimer of ~100 kDa subunits; each R domain contains two GAFs (a and b). GAF*a*, and perhaps GAF*b*, and both provide for allosteric cGMP binding contribute importantly to dimerization. The $K_m$ for cGMP hydrolysis is $<1$ μM, and the $V_{max}$ is ~5 μmol $min^{-1}$ $mg^{-1}$. PDE5 is abundant in smooth muscle cells, gastrointestinal epithelial cells, platelets, and certain neuronal cells. It is typically coexpressed with other components of cGMP signaling, including PKGs and GCs. Catalytic activity is stimulated by cGMP binding to the allosteric cGMP-binding sites, by phosphorylation of Ser-102 near the N terminus, and by chemical reduction. Phosphorylation occurs in intact tissues in response to elevation of cGMP and PKG action.

PDE5 is the major cGMP-hydrolyzing PDE in platelets, lung, and the vascular smooth muscle (VSM) of the penile corpus cavernosum (PCC). Sildenafil (Viagra), tadalafil (Cialis), or vardenafil (Levitra) blocks cGMP breakdown by PDE5 leading to increased intracellular cGMP and relaxation of the VSM of blood vessels within and supplying the PCC. The increased blood flow and capacitance of PCC vascular structures facilitate accumulation of blood and improve erectile function. Sildenafil also shows promise in treatment of other diseases involving the vasculature.

### PDE6

PDE6 occurs only in mammalian retinal photoreceptor cells. Its activity accounts for the visual response. Rods provide for vision in dim light and contain PDE6$\alpha\beta$, a heterodimer of 100-kDa subunits. Cones provide for vision in bright light and contain PDE6$\alpha'\alpha'$, a homodimer of 100-kDa subunits. In the absence of light, PDE6$\alpha\beta$ and PDE6$\alpha'\alpha'$ are inhibited by specific proteins, P$\gamma$s. Activated PDE6 isoforms have high catalytic activity ($V_{max} \sim 300$ μmol $min^{-1}$ $mg^{-1}$) and $K_m \sim 35$ μM. One or both GAFs in each PDE6 subunit provide for allosteric cGMP binding and/or dimerization.

In the dark, cGMP in photoreceptors is high. This cGMP binds to a cGMP-gated cation channel which then allows for increased conductance of a "dark current." When light strikes the retina, the inhibition of PDE6 by P$\gamma$ is relieved, causing a sudden drop in cGMP. At the lower cGMP level cGMP dissociates from the channel; the channel closes, terminating the "dark current." The resulting change in the membrane potential produces the sensation of light. The regulation of PDE6 in cones and rods is specifically adapted to optimally respond to different light intensities, but the overall regulation of the PDE6 isoforms is quite similar.

### PDE9

Little is known about the tissue distribution or physiological role of PDE9. It is highly selective for cGMP and has a $K_m$ of 0.1–2 μM for cGMP versus 230 μM for cAMP. It is inhibited by zaprinast ($IC_{50} \sim 35$ μM), but it is insensitive to a wide range of other PDE inhibitors including sildenafil, vinpocetine, IBMX, and dipyridamole.

### Characteristics of Class I Dual-Specificity PDEs

A number of mammalian PDEs (PDEs 1, 2, 3, 10, and 11) hydrolyze both cGMP and cAMP. The physiological role of each PDE in hydrolyzing the two nucleotides is likely to be complex. Activities of these PDEs towards either cGMP or cAMP will reflect the respective $K_m$ and $V_{max}$ values for each, but selective action towards one nucleotide can occur simply by mass action. For example, if the cGMP/cAMP content of a tissue selectively changes, increased hydrolysis of one nucleotide (e.g., in response to elevated amounts of that nucleotide) would tend to decrease hydrolysis of the other nucleotide through simple competition for the catalytic site. As a result, the concentration of the second nucleotide would increase.

#### PDE1

PDE1 is regulated by $Ca^{2+}$/calmodulin binding (Figure 3). It occurs in many tissues including brain, heart, vascular smooth muscle, and liver. There are three groups of isoforms (PDE1A, IB, and IC) whose relative affinities and turnover rates for cGMP and cAMP vary considerably. In tissues containing primarily one of these isoforms, the relative hydrolytic contribution of PDE1 family to breakdown of cAMP or cGMP will reflect the kinetic features of that particular PDE1. The PDE1 family can provide for responsiveness of cGMP and/or cAMP levels to changes in $Ca^{2+}$ signaling.

#### PDE2

PDE2 is known as the cGMP-stimulated PDE. It is a homodimer comprised of ~105-kD monomers; there are three isoforms. The $V_{max}$ values for hydrolysis of cAMP and cGMP are similar (150 μmol $min^{-1}$ $mg^{-1}$), and the $K_m$ values differ ~twofold (15–30 μM for cGMP versus 30–50 μM for cAMP). Cyclic GMP binds to the GAF b in the R domain; this stimulates breakdown of either cAMP or cGMP. PDE2 can therefore potentially lower either or both cNs. PDE2 is abundant in adrenal cortex cells which produce aldosterone in response to cAMP elevation. Elevation of cGMP results in cGMP binding to the PDE2 GAF b, causing increased cAMP hydrolysis and decreased aldosterone production. A similar physiological effect to increase cGMP hydrolysis has not been experimentally demonstrated, but it is likely to occur.

#### PDE3

PDE3 is abundant in many tissues and occurs as two isoforms (PDE3A and 3B) that are largely membrane bound; both hydrolyze cAMP with a greater $V_{max}$ than cGMP, but the $K_m$ values for the cNs are similar. PDE3B is activated by phosphorylation. Historically, PDE3 was dubbed the "cGMP-inhibited PDE," a misleading descriptor since cGMP "inhibits" cAMP hydrolysis by competing for access to the catalytic site. In platelets, evidence suggests that the antiaggregatory effect of cGMP may occur through a cAMP-signaling pathway due to cGMP competition with cAMP hydrolysis by PDE3. Elevation of cGMP would increase its effectiveness to compete with cAMP for the PDE3 catalytic site, thereby "protecting" cAMP from hydrolysis.

#### PDEs 10 and 11

Little is known about the physiological functions of these recently characterized PDEs. PDE10 is abundant in neural tissue. It has 26-fold higher affinity for cAMP versus cGMP ($K_m$ values ~0.05 and 13 μM, respectively), but the $V_{max}$ for cGMP is ~5 times that for cAMP. PDE10 R domain contains GAFs, but no ligand has been shown to bind to these GAFs. PDE11 is closely related to PDE5 (50% homology). There are four isoforms whose R domains contain one, two, or partial GAFs, but cN binding has not been demonstrated. PDE11 hydrolyzes cGMP and cAMP at a similar rate, but $K_m$ for cAMP is half that for cGMP (0.5 versus 1 μM, respectively). It is potently inhibited by tadalafil (Cialis) ($IC_{50}$ ~ 80 nM) and by dipyridamole ($IC_{50}$ ~ 0.4–0.9 μM).

## Characteristics of Class II PDEs

The few known class II PDEs occur in yeast, bacteria, and *Dictyostelium*. They belong to a family of proteins with a $Zn^{2+}$-binding hydrolase motif that is thought to mediate hydrolysis of the cN. This family of proteins includes glyoxylases, β-lactamases, and arylsulfatases, but there are no X-ray crystallographic structures. There is no apparent homology with class I PDEs.

### Characteristics of Class II PDEs Catalytic Activity

Class II PDEs require divalent cation(s) for catalytic activity. Three of these PDEs hydrolyze cGMP; these include a dual-specificity PDE from *Vibrio fischeri*, and two PDEs from *Dictyostelium* (Figure 4). The *V. fischeri* PDE hydrolyzes cAMP and cGMP with ~20- and 10-fold higher $V_{max}$ values, respectively, than any PDE in either class; one *Dictyostelium* class II PDE is cGMP-specific while the other has dual-specificity.

### Regulation of Class II PDEs

Both *Dictyostelium* PDEs described above are activated by cN binding to site(s) in R domains located near the

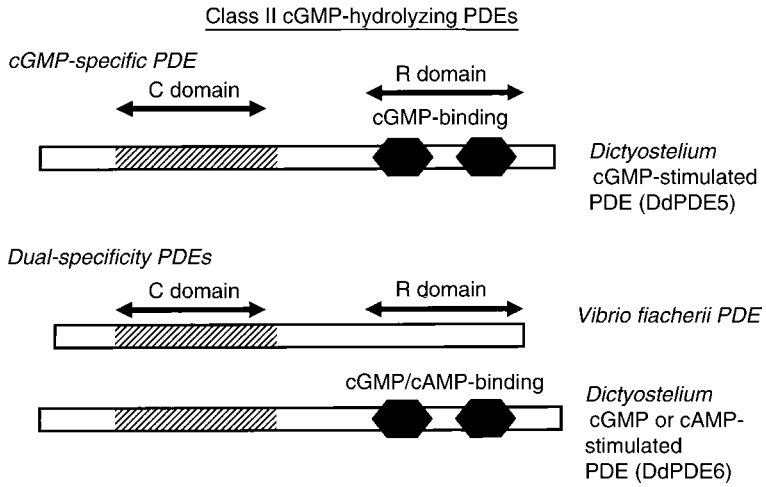



**FIGURE 4** Schematic depiction of class II cGMP-hydrolyzing PDEs. Arrangements of functional domains are shown for a monomer. The allosteric cN-binding sites belong to the CAP-related family of cN-binding proteins and are distinct from the allosteric cGMP-binding sites in class I PDEs.

C terminus (Figure 4). In DdPDE5, catalytic activity is stimulated by allosteric cGMP binding, whereas allosteric binding of either nucleotide stimulates catalysis by DdPDE6. This resembles the cGMP effect on catalysis in class I PDEs 2 and 5. However, GAFs are absent in these class II PDEs. Instead, both contain two CAP-related cN-binding domains (Figure 4). Thus, despite little similarity between class I and II PDEs, similar regulatory mechanisms are used, and, remarkably, evolutionarily distinct motifs and arrangements of the motifs in the structures have been employed. In *Dictyostelium*, cellular cGMP is degraded by both of these PDEs as well as by a dual-specificity PDE that is poorly understood.

## Concluding Remarks

Cyclic GMP levels are modulated through the action of cGMP-specific PDEs and dual-specificity (cGMP and cAMP) PDEs. Both must be considered when studying physiological changes in intracellular cGMP.

### See Also the Following Articles

Cyclic AMP Receptors of *Dictyostelium* • Cyclic Nucleotide Phosphodiesterases • Cyclic Nucleotide-Dependent Protein Kinases • Cyclic Nucleotide-Regulated Cation Channels

### Glossary

**allosteric binding** Binding of ligands to regulatory sites within a protein.

**autoinhibition** Effect of one region of an enzyme to block the action of the catalytic site of another region of that same enzyme.

**CAP-related domains** cN-binding domains of ~120 amino acids that provide for cN binding in PKA, PKG, GEFs, and cN-gated cation channels and are evolutionarily related to the bacterial catabolite-gene activator protein.

**chimeric proteins** Proteins that are composed of multiple subdomains that provide for a specific function of that protein.

**dual-specificity** Proteins that are not entirely selective among closely related ligands, e.g., cAMP and cGMP.

**GAF domains** Domains of ~120 amino acids that provide for binding of a variety of ligands and protein–protein interactions in diverse proteins.

**phosphohydrolase** Enzymes that break a phospho-ester bond.

### Further Reading

Bosgraaf, L., Russcher, H., Snippe, H., Bade, S., Wind, J., and Van Haastert, P. J. M. (2002). Identification and characterization of two unusual cGMP-stimulated phoshodiesterases in *Dictyostelium*. *Mol. Biol. Cell.* **13**, 3878–3889.

Chabre, M., and Deterre, P. (1989). Molecular mechanism of visual transduction. *Eur. J. Biochem.* **179**, 255–266.

Corbin, J. D., and Francis, S. H. (1999). Cyclic GMP phosphodiesterase-5: Target of sildenafil. *J. Biol. Chem.* **274**, 13729–13732.

Francis, S. H., Turko, I. V., and Corbin, J. D. (2000). Cyclic nucleotide phosphodiesterases: Relating structure and function. *Prog. Nucleic Acid Res. Mol. Biol.* **65**, 1–52.

Martinez, S. E., Wu, A. Y., Glavas, N. A., Tang, X.-B., Turley, S., Hol, W. G. J., and Beavo, J. A. (2002). The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding. *PNAS USA* **99**, 13260–13265.

Shakur, Y., Holst, L. S., Landstrom, T. R., Movsesian, M., Degerman, E., and Manganiello, V. (2001). Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family. *Prog. Nucleic Acid Res. Mol. Biol.* **66**, 241–277.

Soderling, S. H., and Beavo, J. A. (2000). Regulation of cAMP and cGMP signaling: New phosphodiesterases and new functions. *Curr. Opin. Cell Biol.* **12**, 174–179.

Sung, B.-J., Hwang, K. Y., Jeon, Y. H., Lee, J. I., Heo, Y.-S., Kim, J. H., Moon, J., Yoon, J. M., Hyun, Y.-L., Kim, E., Eum, S. J., Park, S.-Y., Lee, J.-O., Lee, T. G., Ro, S., and Cho, J. M. (2003). Structure of the catalytic domain of human phosphodiesterase 5 with bound drug molecules. *Nature* **425**, 98–102.

Van Haastert, P. J. M., and Kuwayama, H. (1997). cGMP as second messenger during *Dictyostelium* chemotaxis. *FEBS Lett.* **410**, 25–28.

Xu, R. X., Hassell, A. M., Vanderwall, D., Lambert, M. H., Holmes, W. D., Luther, M. A., Rocque, W. J., Milburn, M. V., Zhao, Y., Ke, H., and Nolte, R. T. (2000). Atomic structure of PDE4: Insights into phosphodiesterase mechanism and specificity. *Science* **288**, 1822–1825.

Yarfitz, S., and Hurley, J. B. (1994). Transduction mechanisms of vertebrate and invertebrate photoreceptors. *J. Biol. Chem.* **269**, 14329–14332.

Zhao, A. Z., Yan, C., Sonnenburg, W. K., and Beavo, J. A. (1997). Recent advances in the study of $Ca^{2+}$/CaM-activated phosphodiesterases. In *Advances in Second Messenger and Phosphoprotein Research: Signal Transduction in Health and Disease*, Vol. 31, pp. 237–251. Lippincott-Raven, Philadelphia.

## Biography

Sharron Francis is a Research Professor in the Department of Molecular Physiology and Biophysics at Vanderbilt University School of Medicine. Her interest has been in the mechanism of action of cGMP and cAMP. She has a Ph.D. in Physiology from Vanderbilt University and did postdoctoral training at Washington University in St. Louis, MO and at the National Heart and Lung Institute at the National Institutes of Health in Bethesda, MD. She has made seminal discoveries in the mechanisms and functions of cGMP-dependent protein kinases and cGMP-binding cGMP-specific phosphodiesterase (PDE5).

Jackie Corbin is Professor in the Department of Molecular Physiology and Biophysics at Vanderbilt University School of Medicine. His career interest has been the mechanism of action of cAMP and cGMP. He has a Ph.D. in Physiology from Vanderbilt University and did postdoctoral training at the University of California-Davis. He has made seminal discoveries in the mechanisms and functions of cAMP-dependent protein kinases, cGMP-dependent protein kinases, and cyclic GMP-binding cyclic GMP-specific phosphodiesterase (PDE5).

# Cyclic Nucleotide Phosphodiesterases

**Vincent C. Manganiello**
*National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA*

**Eva Degerman**
*Lund University, Lund, Sweden*

**Cyclic nucleotide phosphodiesterases (PDEs) constitute a large, diverse, and complex superfamily of metallohydrolases which cleave the 3′,5′-cyclic phosphate bond of cyclic AMP (cAMP) and cyclic cGMP (cGMP), resulting in production of 5′-AMP and 5′-GMP, respectively. Based on differences in primary structures, PDEs have been divided into two major classes, I and II. Class I PDEs, which contain a conserved catalytic domain (~250–300 amino acids), comprise the majority of known PDEs, including the 11 structurally related, highly regulated, and functionally distinct mammalian gene families (PDEs 1–11). Class I PDEs are also found in the parasites *Trypanosoma brucei* and *cruzi*, and in *Drosophila*, nematodes, yeast, and sponges. Only a few enzymes (no mammalian isoforms) have been classified as class II PDEs, which are structurally unrelated to class I PDEs. The evolutionary relationship between the two PDE classes is also not certain; this brief review will focus on mammalian class I PDEs. By catalyzing hydrolysis of cyclic nucleotides, mammalian PDEs regulate their intracellular concentrations, and, consequently, their signaling pathways and myriad physiological effects, including myocardial contractility, visual transduction, vascular and airway smooth muscle relaxation, immune/inflammatory responses, cell proliferation and apoptosis, memory, and many others. At present, mutations in the PDE6 gene represent the only known association of PDE mutations with human disease, in this instance, with certain subclasses of degenerative retinitis pigmentosa. The mammalian PDE superfamily is a major target for drug discovery in treatment of clinically important diseases, i.e., PDE5 inhibitors (Viagra®) for erectile dysfunction, PDE4 inhibitors for inflammatory disorders, etc.**

## Molecular Diversity

cAMP and cGMP are important intracellular second messengers that modulate many biological processes. The "classical" mechanism for transduction of cyclic nucleotide signals involves cyclic nucleotide-induced activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively), with subsequent phosphorylation of critical downstream regulatory effectors. Recently, however, cyclic nucleotide-binding proteins have been recognized as direct mediators of cyclic nucleotide actions, e.g., cyclic nucleotide-gated ion channels, cAMP-activated guanine nucleotide exchange factors (EPACs) which regulate Rap1 GTPases (guanosine triphosphatases), and several PDEs, especially PDEs 2, 5, 6, which contain allosteric, non-catalytic cyclic nucleotide-binding sites.

Molecular genetics has revealed the diversity and complexity of the 11 mammalian PDE gene families (PDEs 1–11). PDE families differ in their primary amino sequences, substrate specificities, sensitivities to endogenous effectors and pharmacological agents, cellular functions, and mechanisms whereby they are regulated. Most PDE families comprise more than one gene; within these families multiple, closely related isoforms are generated from the same gene or different genes via alternative mRNA splicing or utilization of different promoters/transcription initiation sites. More than 20 PDE genes probably encode more than 50 PDE proteins. Some cells are relatively enriched in specific PDEs, e.g., photoreceptor PDE6 is virtually exclusively expressed in the retina. Most cells, however, contain representatives of multiple PDE gene families, and different members of the same family, but in different amounts, proportions and subcellular locations. Redundancy in PDEs, i.e., the presence in the same cell of multiple enymes which essentially perform the same reaction of hydrolyzing cyclic nucleotides, does not merely serve a survival or protective function, but rather allows cells to use distinct subsets of differentially regulated and localized PDEs to specifically and selectively regulate and segregate the generation, amplitude, duration, and compartmentation of cyclic nucleotide signals and actions. It is generally accepted that the cellular capacity to degrade cyclic nucleotides far

exceeds their synthesis, and is a major factor in the very rapid turnover of intracellular cAMP and cGMP. The physiological significance and functional consequences of this rapid turnover, and concomitant heat generation (cyclic nucleotide hydrolysis is accompanied by release of $\sim 7 \text{cal mol}^{-1}$), are not understood. This brief review will discuss some general characteristics of PDEs and then focus on the cellular biology and diverse functions of different PDE isoforms and their potential as therapeutic targets.

## Structure/Function Analyses

### Catalytic Domain

Mammalian PDEs exhibit a common structural organization, with a conserved catalytic domain ($\sim$250–300 amino acids) in the C-terminal portion of the molecules and divergent regulatory domains and modules in N-terminal portions (Figure 1). The catalytic core, more highly conserved among members of the same gene family than different gene families, contains a signature motif [HD ($X_2$) H ($X_4$) N], common to all PDEs, and includes consensus metal-binding domains. In addition to common structural elements responsible for cyclic nucleotide hydrolysis, the catalytic core contains family specific sequences responsible for differences in substrate affinities, catalytic properties, and sensitivities to specific inhibitors (Figure 1). Some PDE families are relatively specific for hydrolysis of cAMP (PDEs 4, 7, 8); others, for cGMP (PDEs 5, 6, 9); and some exhibit mixed specificity for both cAMP and cGMP (PDEs 1, 2, 3, 10, 11). While methylxanthines inhibit almost all PDEs, relatively specific and selective inhibitors, i.e., drugs that target individual PDE families with 10–100-fold greater potency than other PDE families, are available for several families, e.g., PDEs 1, 2, 3, 4, 5, 6. These family specific inhibitors are important for both basic research and clinical applications.

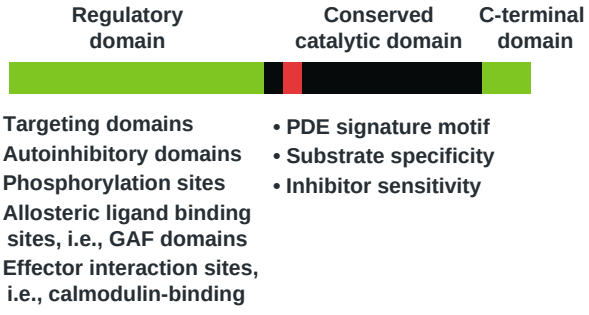



**FIGURE 1** Common structural pattern for different PDE gene families.

### Regulatory Domain

N-terminal portions of PDE molecules are highly divergent, containing structural determinants and specific amino acid sequences that allow different PDEs to respond selectively to specific regulatory signals (Figure 1). These regulatory regions include autoinhibitory modules (e.g., in PDEs 1, 4, 5), as well as sites and domains that are subject to different types of covalent modification (e.g., sites (in PDEs 1, 3, 4, 5, 10, 11) for phosphorylation by various protein kinases), or that interact with allosteric ligands (e.g., cGMP-binding sites in GAF domains), specific effectors (e.g., $Ca^{2+}$/calmodulin), protein partners or molecular scaffolds [e.g., AKAPs (anchoring proteins for PKA), B-arrestin], and thereby regulate catalytic activity, protein–protein interactions and/or subcellular compartmentation and localization (Figure 1). Five PDE families (PDEs 2, 5, 6, 10, 11) contain homologous so-called GAF domains, an acronym for proteins (cGMP-binding PDEs, *A*nabena adenylyl cyclase and *f*hlA, an *E. coli* transcriptional regulator) that contain these sequences. Although cGMP binding is not the primary function of GAF domains, in three PDE families (PDEs 2, 5, and 6), GAF domains bind cGMP with high affinity, but with different functional consequences.

## PDE Functions

### PDEs: Components of Spatially Organized Signaling Networks and Microdomains

PDEs are critical regulators of the generation, amplitude, duration, and termination of intracellular cyclic nucleotide signals. In addition to tight regulation of their concentrations and turnover, intracellular pools of cAMP and cGMP are also temporally, spatially, and functionally compartmentalized. In cardiac myocytes, for example, $PGE_1$ and catecholamines increase cAMP and activate PKA in different compartments, and cAMP diffusion is spatially restricted and regulated, at least in part, by PDEs. Newer techniques, e.g., use of fluorescence-resonance energy transfer (FRET) and cyclic nucleotide biosensors, also suggest that PDEs play an important role in these spatially-constrained microdomains, i.e., discrete subcellular regions in which cyclic nucleotide gradients are generated, monitored, and regulated, and their signals and effects channeled, transduced, and modulated.

The molecular basis for compartmentalization of cAMP signaling involves anchoring of PKA isoforms at specific intracellular sites via AKAP scaffolding proteins. These proteins organize formation of localized signaling modules/microdomains consisting of kinases, kinase

substrates, phosphatases, PDE4 isoforms, and other proteins. These modules sense intracellular cAMP gradients and effectively compartmentalize activation of substrates and biological responses. In these microdomains, cAMP-induced activation of PKA also results in phosphorylation/activation of PDE4 isoforms associated with AKAPs, resulting in modulation and termination of cAMP signals, and return of PKA to its basal state. For example, in cardiac myocytes, PDE4 isoforms are associated with AKAP complexes thought to modulate effects of cAMP on L-type and ryanodine-sensitive $Ca^{2+}$ channels.

PDE4 isoforms, PKA, and other molecules also interact/associate with $\beta$-arrestins, which are scaffolding proteins located in the vicinity of plasma membranes that interact with activated $\beta$ receptors and are involved in coordination of $\beta$ receptor signaling and trafficking. This targeting and effective concentrating of PKA and PDE4 in proximity to the activated $\beta$ receptors not only reduces cAMP generation (via phosphorylation, uncoupling and internalization of $\beta$ receptors) but also enhances localized degradation of cAMP and desensitizes the complex with respect to further signaling via the $\beta$ receptor and cAMP.

The subcellular localization of different PDEs, their interactions with molecular scaffolds and interacting partners, and their inclusion in, and contribution to the function of, macromolecular signaling microdomains is becoming a common theme in PDE biology. Anchoring of PDE3B to intracellular membranes via transmembrane hydrophobic helical segments and its interaction with PKB may be important in its activation by insulin. PDE4s also associate, via an N-terminal helical region, with another signaling scaffold protein called RACK1 (receptor for activated protein kinase C 1).

## PDEs: Intracellular Effectors

PDEs are not only important determinants in the stringent regulation of intracellular cAMP and cGMP concentrations but they also serve as effectors of cyclic nucleotide actions. Historically, in cells containing multiple PDEs, family specific PDE inhibitors were initially utilized to pharmacologically define roles of specific PDEs in regulating specific signaling pathways and discrete cellular functions. In cultured mammalian oocytes, PDE3 inhibitors, not PDE4 or 5 inhibitors, inhibit meiotic progression and oocyte maturation, implying that PDE3 regulates a cAMP pool that controls maturation. (In fact, female PDE3A KO mice are sterile.) In cultured renal mesangial cells, experiments with PDE3 and PDE4 inhibitors indicated that PDE3 and PDE4 selectively regulated functionally distinct cAMP pools that controlled cell growth, and generation of reactive oxygen species, respectively. These types of studies first suggested a role for PDEs in spatial and/or functional compartmentalization of cyclic nucleotide signals and effects.

In some instances, a specific PDE serves as a critical effector system and regulates a unique cellular function, e.g., in retinal rods and cones, light-induced activation of photoreceptor PDE6 results in hydrolysis of cGMP and initiation of visual signal transduction. Activation of PDE3s by insulin, IGF-1, and leptin is apparently important in their ability to reduce cAMP and thereby regulate lipolysis in adipocytes, glycogenolysis in hepatocytes, insulin secretion from pancreatic $\beta$ cells, and oocyte maturation. In these instances, PDE3 inhibitors can attenuate or block the effects of insulin, IGF-1 and leptin.

Studies of PDE KO mice do indicate that the ability of specific PDEs to regulate discrete cAMP/cGMP signaling pathways and actions is genetically determined. Female PDE3A KO mice are sterile, most likely due to inhibitory effects of cAMP on meiotic progression and maturation of oocytes, and consequently, their competency for fertilization. PDE3B KO mice, not frankly diabetic, demonstrate signs of disruption in insulin secretion and insulin resistance. Production of TNF$\alpha$ in response to administration of lipopolysaccharide (LPS) in PDE4B KO mice is profoundly ($\sim$90%) inhibited. On the other hand, PDE4D KO mice do not exhibit acetylcholine-induced contraction and hyperreactivity of airway smooth muscle. In PDE4D KO mice, the duration of $\beta$-adrenergic-induced anesthesia is reduced by more than 50%, and fertility is decreased due to reduced ovulation.

## PDEs: Signal Integrators

With their different intrinsic properties and different responses to regulatory signals, PDEs also integrate multiple inputs, and are a "locus" for crosstalk between different signaling pathways. For example, PDE2, which is allosterically activated by cGMP, is highly concentrated in adrenal zona glomerulosa cells. In these cells, ANF (atrial natriuretic factor) increases cGMP synthesis; cGMP, in turn, activates PDE2, leading to a decrease in cAMP and PKA activity and inhibition of cAMP-stimulated aldosterone production. On the other hand, in other cells, NO activates guanylyl cyclase and increases cGMP, which inhibits PDE3 and increases intracellular cAMP content, resulting, for example, in stimulation of renin secretion from the juxtaglomerular apparatus in the kidney, and inhibition of platelet aggregation.

Newer approaches have suggested that different PDE isoforms may not only coordinate regulation of signals and responses in individual cells, but that differential cellular distribution within tissues may be important in intercellular communication and regulation of tissue

function. Immunohistochemical studies indicate, for example, that PDE1C and PDE2A are expressed in different sets of neurons in the olfactory epithelium, whereas PDE1C and PDE4A are present in different subcellular regions of the same neurons.

### PDEs: Homeostatic Regulators

Signaling pathways, in general, also include mechanisms for negative feedback control. PDE3 and PDE4 activities, for example, are acutely up-regulated by cAMP-induced activation of PKA, which results in phosphorylation/activation of PDE3 and PDE4, and enhanced destruction of cAMP. Chronic elevation of cAMP also provides negative feedback, by increasing transcription of PDE3 and PDE4 genes and protein synthesis, resulting in increased enzymatic activities. In some cells, this latter phenomenon is part of mechanisms involved in ligand-induced down-regulation of cellular responses, such as tachyphylaxis or desensitization. Negative feedback control of cGMP hydrolysis by PDE5 is somewhat different, due to the presence of high affinity, non-catalytic, allosteric-binding sites for cGMP in the GAF domains of PDE5. Binding of cGMP to GAF domains results in allosterically-induced conformational changes that increase affinity of the catalytic site for cGMP and also facilitates phosphorylation and activation of PDE5 by PKG. Thus, elevation in intracellular cGMP provides negative feedback control and enhances its own destruction, both via direct cGMP-induced allosteric activation as well as indirect activation due to phosphorylation of PDE5 by PKG.

## Family Specific Inhibitors: Clinical Applications

Molecular diversity of PDEs has occasioned the development of family specific PDE inhibitors to replace nonselective PDE inhibitors (theophylline and caffeine) as therapeutic agents for diseases such as asthma. Despite intensive efforts to develop such drugs, only sildenafil (Viagra™), a selective PDE5 inhibitor, has proven to be therapeutically effective in a major disease, i.e. erectile dysfunction, and is showing promise as a treatment modality in pulmonary hypertension. PDE4 enzymes are relatively concentrated in immume/inflammatory cells, and specific PDE4 inhibitors exhibit potent anti-inflammatory actions. Two selective PDE4 inhibitors, cilomilast and rofumilast, are in Phase 3 clinical trials for treatment of chronic obstructive pulmonary disease (COPD) and asthma. PDE3 inhibitors, which enhance myocardial contractility and relaxation of smooth muscle and inhibit platelet aggregation, failed in long-term clinical trials for treatment of chronic heart failure. However, one PDE3 inhibitor, milrinone, is used for acute and short term treatment of adult patients with decompensated and refractory cardiac failure, and another, cilostazol, has FDA approval for treatment for intermittent claudication.

## Conclusions

By virtue of their distinct intrinsic characteristics and differential regulation, their intracellular targeting to different subcellular locations and microdomains, and their interactions with cellular structural elements, regulatory partners, and molecular scaffolds such as AKAPs and $\beta$ arrestin, different PDEs can integrate multiple cellular inputs, and modulate the intracellular diffusion and functional compartmentalization of cyclic nucleotide signals. The combined enormous molecular diversity of receptors and their ligands, adenylyl and guanylyl cyclase systems, PDEs, and cyclic nucleotide-regulated effector systems, coupled with their physical and functional compartmentalization, provides for the complex integration, specificity, and variety of networks and pathways involved in generation, transduction, modulation, and termination of cyclic nucleotide-gated signals and actions. The ability of scaffolding proteins to spatially organize signaling molecules, including protein kinases, phosphatases, kinase substrates, PDEs, and other signaling and effector molecules, effectively allows cells to generate signaling specificity by using small, discrete subsets of proteins which exhibit overlapping or redundant functions. Integration of these myriad combinations establishes the unique cyclic nucleotide networks and phenotypes that characterize individual cells.

### See Also the Following Articles

Adenylyl Cyclases • A-Kinase Anchoring Proteins • Cyclic GMP Phosphodiesterases • G Protein-Coupled Receptor Kinases and Arrestins

### Glossary

**cyclic nucleotide phosphodiesterases (PDEs)** Enzymes that catalyze hydrolysis of the 3′-5′-phosphodiester bond of cAMP and/or cGMP.

**desensitization** Prolonged or repeated exposure of receptors to their ligands leads to reduction or loss of responsiveness, either homologous desensitization with loss of responsiveness only to a specific ligand or heterologous desensitization with loss of responsiveness to multiple ligands.

**family specific inhibitors** Drugs that inhibit the activity of one PDE gene family with 10–100-fold greater potency than the activities of other PDE gene families.

## Further Reading

Beavo, J., and Brunton, L. (2002). Cyclic nucleotide research—still expanding after half a century. *Nat. Rev., Molecul. Cell Biol.* **3**, 710–718.

Conti, M., Richter, W., Mehats, C., Livera, G., Park, J.-Y., and Jin, C. (2003). Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J. Biol. Chem.* **278**, 5493–5496.

Degerman, E., Rahn-Landstrom, T., Stenson-Holst, L., Goranssen, O., Harndahl, L., Ahmad, F., Choi, Y.-H., Masciarelli, S., Lui, H., and Manganiello, V. C (2003). Role for phosphodiesterase 3B in regulation of lipolysis and insulin secretion. In *Diabetes Mellitus: A Fundamental and Clinical Text* (D. LeRoith, S. I. Taylor, and J. M. Olefsky, eds.), 3rd edition. Lippincott-Raven Publishers, Philadelphia, PA, Ch. 24, pp. 373–381.

Francis, S. H., Turko, I. V., and Corbin, J. D. (2001). Cyclic nucleotide phosphodiesterases—relating structure and function. *Prog. Nucleic Acid Res. Mol. Biol.* **65**, 1–52.

Houslay, M. D., and Davis, D. R. (2003). PDE4 cAMP phosphodiesterase: Modular enzymes that orchestrate signaling crosstalk, desensitization and compartmentalization. *Biochem. J.* **370**, 1–18.

Manganiello, V. C., and Degerman, E. (1999). Cyclic nucleotide phosphodiesterases (PDEs): Diverse regulators of cyclic nucleotide signals and inviting molecular targets for novel therapeutic agents. *Thrombosis and Haemostasis* **82**, 407–411.

Mehats, C., Andersen, C., Filopanti, M., Jin, S.-L. C., and Conti, M. (2002). Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. *Trends Endocr. Metab.* **13**, 29–35.

## Biography

Eva Degerman received her degrees, M.D. in 1991 and Ph.D. in 1988, from Lund University, where she has spent her entire scientific career.

Vincent Manganiello received his degrees, M.D. and Ph.D., from Johns Hopkins University School of Medicine in 1967. After serving as a pediatric intern in the Harriet Lane Service at Johns Hopkins, he came to the National Institutes of Health in 1968, and has pursued his entire scientific career at the National Heart, Lung, and Blood Institute. Drs. Degerman and Manganiello have enjoyed a long-standing collaboration focused on the cyclic nucleotide phosphodiesterase 3 (PDE3) family and its role in metabolic regulation and insulin secretion in adipose tissue, liver and pancreas, important target tissues for actions of insulin, leptin and IGF-1 (insulin-like growth factor-1), and for maintenance of energy homeostasis, as well as for the pathogenesis of type 2 diabetes and obesity.

# Cyclic Nucleotide-Dependent Protein Kinases

**Sharron H. Francis and Jackie D. Corbin**
*Vanderbilt University School of Medicine, Nashville, Tennessee, USA*

**The cyclic nucleotide-dependent protein kinases, cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), are the major intracellular receptors for adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP), respectively. Upon elevation of cAMP or cGMP in the cell, these nucleotides can bind to numerous cellular proteins including the cyclic nucleotide-dependent protein kinases. Intracellular levels of cAMP and cGMP are determined by the balance between their synthesis and breakdown. They are synthesized from either ATP or GTP by adenylyl cyclase or guanylyl cyclase, respectively, in response to first messengers in the body (e.g., hormones, neurotransmitters, and various environmental stimuli), and they are broken down by cyclic nucleotide phosphodiesterases (PDEs). The important concept of signaling through second messengers such as cAMP and cGMP is that the hormone, neurotransmitter, or other stimuli can elicit their physiological effects without entering the cell. The protein kinase catalytic activities of cyclic nucleotide-dependent kinases are activated when the cyclic nucleotides bind to their regulatory domains, and the catalytic domains of the kinases can then transfer a phosphate from ATP to many proteins through a process known as phosphorylation. The phosphorylation of these target proteins frequently alters their functions and accounts for most of the known effects of cyclic nucleotides in eukaryotes.**

## Cyclic Nucleotide-Dependent Protein Kinases as Intracellular Receptors for cAMP and cGMP

The cyclic nucleotide-dependent protein kinases, cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), are the major intracellular receptors for adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP), respectively (Figure 1). PKA and PKG are homologous ligand-activated kinases. In the presence of very small amounts of cAMP or cGMP (e.g., concentrations ranging from $10^{-8}$–$10^{-7}$ M), these kinases are activated by binding the cyclic nucleotide. The intracellular levels of cAMP and cGMP are determined by the balance between their synthesis and breakdown. They are synthesized from either ATP or GTP by adenylyl cyclase or guanylyl cyclase, respectively, in response to first messengers in the body (e.g., hormones, neurotransmitters, and various environmental stimuli), and they are broken down by cyclic nucleotide phosphodiesterases (PDEs) (Figure 2). The activated PKA or PKG then catalyzes transfer of the $\gamma$-phosphate of ATP to selected serine or threonines in many cellular proteins through a process known as phosphorylation. The covalent attachment of phosphate to these proteins converts them into phosphoproteins, a process known as heterophosphorylation because in this case PKA or PKG phosphorylates proteins other than itself. The phosphoproteins frequently have changes in their activities, subcellular localizations, and interactions with other cellular components, including other proteins, DNA, RNA, and lipids. The altered function of these proteins that have been phosphorylated by PKA or PKG mediates the changes induced by many hormones and environmental stimuli, including those in metabolism, gene transcription, neurotransmission, blood pressure, and fluid homeostasis.

## Overall Structure of Cyclic Nucleotide-Dependent Protein Kinases

There are several isoforms of PKA and PKG, but all have a similar organization of their functional domains (Figure 3). Both are chimeric proteins comprising a regulatory domain and a catalytic domain, and within each of these there are several subdomains that provide for specific functions. PKA regulatory and catalytic domains are located on separate subunits, the R subunit and C subunit, respectively. In PKG, the regulatory and catalytic domains are on a single polypeptide chain. Both enzymes form dimers through interactions between

 © 2004, Elsevier Inc. All Rights Reserved. 

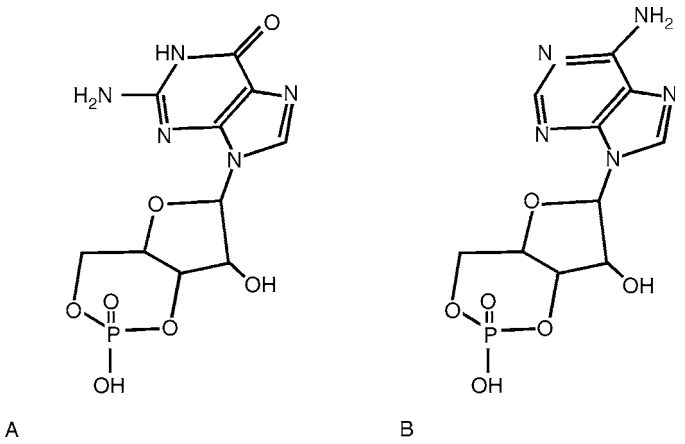



**FIGURE 1** Structural models of (A) cGMP and (B) cAMP. cGMP and cAMP differ only in their purine rings. These differences in the purine ring provide for the specificity with which each kinase interacts preferentially with that nucleotide. The cyclic phosphate moiety is required for binding to the cyclic nucleotide-binding sites on PKG and PKA, and the 2′–OH of the ribose provides an important contact in the respective sites. There are two conformations of these nucleotides, *syn* (shown) and *anti*, due to rotation around the bond linking the purine and the ribose. Both PKA and PKG bind the *syn* conformer. Important features of cGMP that contribute to its interaction with PKG include the amino group at C-2 (which interacts with a conserved threonine in sites preferring cGMP), the oxygen at C-6, and a protonated N-1. cAMP has a single substitution on the purine (i.e., an amino group at C-6 and no protonation at N-1).

amino acids near the N terminus within their regulatory domains. However, the salient biochemical features of regulation and catalysis are retained in monomeric forms of PKG and in PKA formed by the combination of a single R subunit and C subunit.

Although dimerization is not necessary for PKA function, it is required for anchoring a portion of the PKA to specific subcellular compartments through a family of proteins known as A kinase anchoring proteins (AKAPs). Anchoring is thought to bring PKA into close proximity with substrates and to thereby facilitate the rapidity and efficiency of cAMP signaling. PKA R

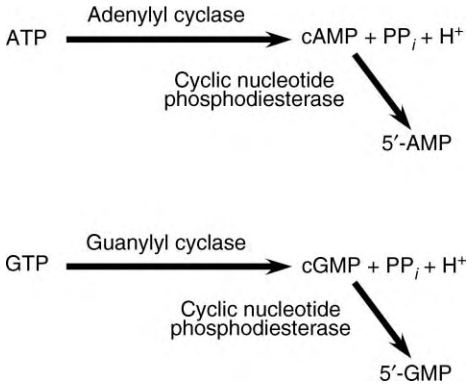



**FIGURE 2** The balance between cyclase and phosphodiesterase activities determines cyclic nucleotide levels in the cell. cAMP and cGMP are synthesized from either (A) ATP by adenylyl cyclase or (B) GTP by guanylyl cyclase; when the activities of these cyclases are increased, more cAMP or cGMP is produced. The breakdown of the cyclic nucleotides is catalyzed by cyclic nucleotide phosphodiesterases (PDEs), and the activities of these enzymes lower cAMP and cGMP. The balance between the activities of these two families of enzymes is the major factor in determining the cellular content of cyclic nucleotides.

subunits are aligned in an antiparallel arrangement within the dimer and use hydrophobic interactions, whereas the PKG monomers within the dimer are aligned in a parallel arrangement through interactions of an extended leucine zipper motif.

## PKA

PKA is present in the cytosolic and particulate fraction of all mammalian tissues and occurs at concentrations ranging from 0.2 to 2 μM. It is a tetramer comprising two C subunits and two R subunits ($R_2C_2$) (Figure 3). There are three main genes for mammalian C subunit (C$\alpha$, C$\beta$, C$\gamma$); each is ~36,000 Da. There are two major genes for R subunits (RI and RII) and subtypes within these (RI$\alpha$, RI$\beta$, RII$\alpha$, and RII$\beta$) are produced by mRNA splicing; each is ~45,000 Da. The nomenclature for PKAs is based on the R subunits; PKAs containing RI subunits are called type I PKA, and those containing RII subunits are called type II PKA. Both types may contain either form of C subunit. Most tissues contain both types of PKA and, in most instances, either type can carry out cAMP-mediated action. Type I PKA is predominantly cytosolic; type II PKA is found in both the cytosol and membrane compartments. The RI and RII content of a particular tissue varies among species.

In the absence of cAMP, an R subunit interacts with a C subunit with high affinity (~0.2 nM) and suppresses its catalytic activity through a process known as autoinhibition. cAMP binding to two cAMP-binding sites on an R subunit causes a conformational change that decreases the affinity of the R subunit for a C subunit by 10,000- to 100,000-fold. An active

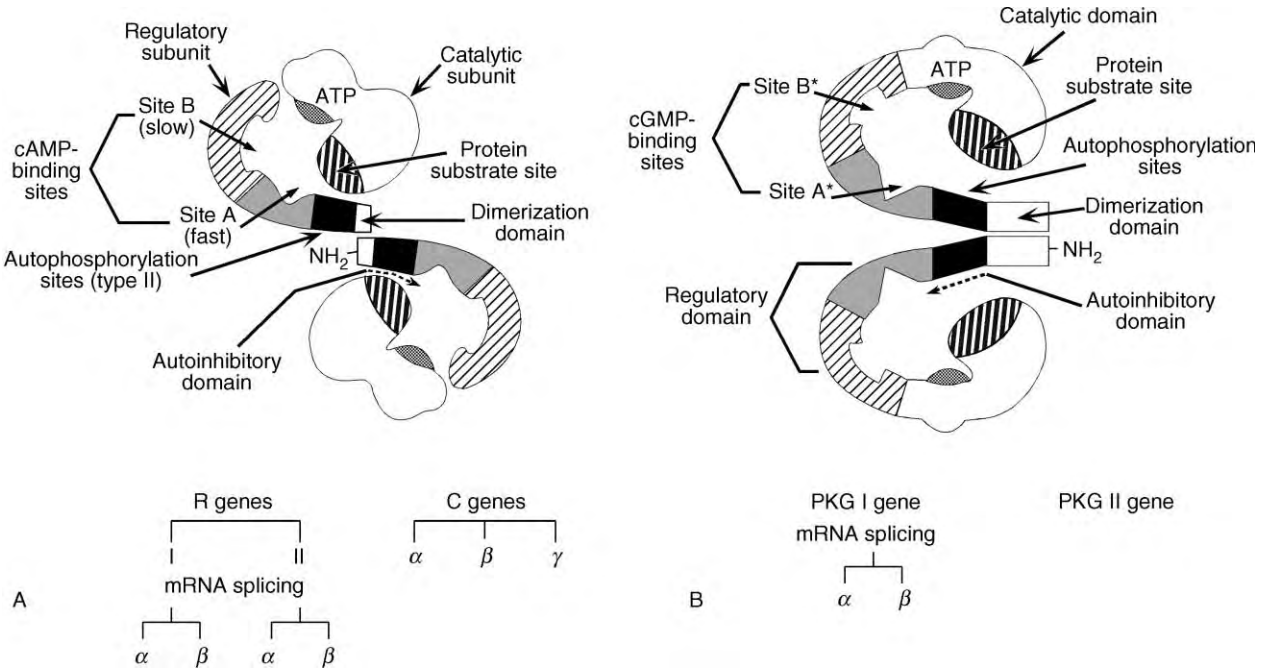



**FIGURE 3** Working models of (A) PKAs and (B) PKGs, depicting the overall organization of the functional domains within these enzymes. The major isozymic forms that are products of different genes for each of the kinases are listed below each model. Additional forms of the PKA R subunits and some of the C subunits are produced by alternative mRNA splicing. The alignment of the low-affinity and high-affinity cyclic nucleotide sites within PKA R subunit is the same for all known PKAs. The linear arrangement of the cGMP-binding sites in PKG-II is the same as that found in PKAs. However, in PKG-I, the sites are transposed so that the more N-terminal site is the high-affinity cGMP-binding site and at the more C-terminal position is the low-affinity site.

monomeric C subunit dissociates from the complex and can diffuse throughout the cell, including the nucleus, which excludes R subunits and PKA holoenzymes. The diffusibility of the C subunit is believed to provide for the mechanism by which cAMP elicts its effect in a number of systems. The R subunits remain dimerized and, if anchored to AKAPs, they remain anchored.

X-ray crystallographic structures have been determined for the C subunit and also for the RI and RII subunits, but to date there is no structure for the inactive complex. The C subunit has two lobes that combine to form a catalytic cleft where the phosphate is transferred from ATP to the protein substrate. The smaller, more N-terminal lobe makes most of the contacts with $Mg^{2+}$/ATP and the larger, more C-terminal lobe makes most of the contacts that precisely position the protein substrate in the catalytic cleft. The binding of $Mg^{2+}$/ATP and a protein substrate into the catalytic cleft allows a complex set of enzymatic actions to transfer the phosphate from ATP to substrate.

In order to efficiently transfer the phosphate to a peptide or protein, PKA strongly prefers an amino acid sequence of -ArgArgXSer/ThrX- (called a PKA consensus phosphorylation site), and this sequence alone provides in large part for the efficiency with which the C subunit phosphorylates a protein. The amino acids in the positions marked as X affect the affinity of the C-subunit interaction with a substrate and, although arginines (Arg) are highly preferred in this motif, some variation is tolerated.

Each R subunit contains several subdomains. These include an N-terminal dimerization subdomain, a subdomain that includes the autoinhibitory and autophosphorylation regions, and a cyclic nucleotide-binding subdomain that contains two cAMP-binding sites arranged in tandem (Figure 3). In the absence of cAMP, a substrate-like sequence in the R subunit binds tightly to the catalytic site in the C subunit. Although other contacts also contribute to stabilizing the R–C interaction, this substrate-like sequence in the autoinhibitory domain competes with protein substrate binding and provides a major portion of the interactions that hold PKA in a catalytically inactive form in the absence of cAMP. This is known as autoinhibition. The binding of cAMP to both cAMP-binding sites in each R subunit disrupts the R–C subunit interaction.

Most commonly, the PKA tetramer contains a single type of R subunit (either RI or RII). RII subunits contain a sequence in the autoinhibitory domain that mimics a PKA substrate motif, and the serine in this sequence can be autophosphorylated by the C subunit when the subunits are complexed (i.e., in the absence of cAMP). Autophosphorylation of this site in RII increases its affinity for cAMP so that PKA can be activated at lower cAMP concentrations. Type I R subunits contain a pseudosubstrate sequence (-ArgArgXGlyX-), that is, a

substrate-like sequence that lacks a phosphorylatable residue. Therefore, type I PKA R subunits cannot undergo autophosphorylation.

PKA plays a critical role in functions of all mammalian cells. As a result, it is likely that survival requires the presence of the PKA C subunit. A null mutation for C subunit in yeast is lethal. Mice carrying null mutations for either C$\alpha$ or C$\beta$ are viable, but differ phenotypically; mice lacking either RI or RII subunits are also typically viable and differ phenotypically. These results suggest that these isoforms of the C subunit and R subunit have some selectivity in function.

### PKG

The tissue distribution of PKG is more restricted than that of PKA. PKGs play a central role in regulating smooth muscle tone, platelet aggregation, bone growth, and water–salt homeostasis. Cellular concentration of PKG ranges from infinitesimally low to 1 μM. There are two families of PKG (PKG-I and PKG-II) that are the products of separate genes. These families are not co-expressed and appear to have selective functions because the phenotype of PKG-I-null mice differs from that of PKG-II-null animals. PKG-I has two alternative splice variants (PKG-I$\alpha$ and PKG-I$\beta$) that vary in the N terminal ~100 amino acids and are typically co-expressed. PKG-I is largely cytosolic, whereas PKG-II is membrane-bound through an N-terminal myristyl group. In some instances, this provides for specific phosphorylation events that do not occur when PKG-II is in its soluble form. The membrane association of PKG-II appears to colocalize PKG-II with the intended substrate. The PKG-I isoforms, PKG-I$\alpha$ and PKG-I$\beta$, are largely cytosolic, but they too are specifically localized in some cases. When this occurs, it involves interactions between residues within the conserved leucine zipper motif near the N terminus and another protein, for example, the myosin-binding protein of the myosin phosphoprotein phosphatase. In many cases, the interaction is selective for either PKG-I$\alpha$ or PKG-I$\beta$, indicating that the specificity of the interaction involves sequences that are novel to one or the other isoform.

PKG regulatory domains contain several subdomains including an N-terminal dimerization subdomain involving a leucine zipper motif, a subdomain containing both the autoinhibitory and autophosphorylation regions, and a cyclic nucleotide-binding subdomain that contains two cGMP-binding sites arranged in tandem (Figure 3). The role of dimerization is unclear because a monomeric PKG retains the salient features of the dimer.

The autoinhibitory–autophosphorylation subdomain is located just C-terminal to the dimerization domain. This region accounts for autoinhibition of the catalytic site and contains multiple autophosphorylation sites, most of which do not resemble a consensus substrate sequence and are not conserved among PKGs. These sites are autophosphorylated by the catalytic domain within the same PKG monomer; autophosphorylation of PKG-I increases the affinity for cGMP and elevates basal activity. Unlike that for PKA, PKG autophosphorylation increases in the presence of cyclic nucleotide.

The mechanism of autoinhibition of PKGs also differs somewhat from that of PKA. The autoinhibitory domain of all PKGs contains either pseudosubstrate sequences, for example, -LysArgGln*Ala*Ile- in PKG-I$\beta$, or sequences that only weakly mimic a pseudosubstrate site, for example, -ArgAlaGln*Gly*Ile- in PKG-I$\alpha$, where the italicized residue indicates the phosphorylation position in the autoinhibitory PKA sequence. Furthermore, sequences outside the pseudosubstrate sequence contribute more prominently to the autoinhibition of both PKG-I and PKG-II compared to PKA.

PKGs contain two cGMP-binding sites of ~110 amino acids arranged in tandem. A small segment of protein connects the regulatory and catalytic domains of the enzyme. The catalytic domain is thought to resemble that of PKA because the enzymes are homologous. In the absence of cyclic nucleotide, PKG catalytic activity is latent. cGMP binding to the cGMP-binding sites in the regulatory domain causes an elongation of the PKG monomer that relieves the autoinhibition, thereby activating catalytic activity.

## Cyclic Nucleotide-Binding Specificity and Affinity

The two homologous cyclic nucleotide-binding sites (~110 amino acids each) in PKA and PKG appear to be the products of an ancient gene duplication that occurred prior to the divergence of these proteins. These sites are evolutionarily related to a bacterial cAMP-binding protein, the catabolite-gene-activating protein (CAP) family of cyclic nucleotide-binding proteins. The cyclic nucleotide-binding sites of cyclic nucleotide-gated channels and cAMP-regulated guanine nucleotide exchange factors (GEFs) are also members of this family. In both PKA and PKG, the two intrasubunit sites differ approximately 10-fold in their affinity for cyclic nucleotide binding and also differ in specificity for cyclic nucleotide analogues. In PKA RI and RII subunits, the more N-terminal site has a lower affinity for cAMP (fast cAMP dissociation) compared to the more C-terminal site (slow cAMP dissociation) (Figure 3). In PKG-II, the cGMP-binding sites are arranged as in PKA, but in PKG-I the higher affinity site is the more N-terminal site. Full activation of either PKA or PKG requires saturation of all the cyclic nucleotide-binding sites.

PKA and PKG have a 50- to 200-fold selectivity for cAMP and cGMP, respectively, and most commonly the

cellular effect of cAMP or cGMP is mediated by the respective kinase. However, in several systems, cAMP or cGMP has been shown to activate the other kinase through a process known as cross-activation. In the X-ray crystallographic structure of the R subunit, cAMP is bound in a deep pocket within each site, and several conserved amino acids (glutamate, arginine, and several glycines) provide direct contact with the cyclic nucleotide or contribute to critical structural features of the site. In sites preferring cGMP, an invariant threonine provides a major portion of the selectivity for cGMP by interacting with the 2′-amino group in the purine (Figure 1). Hydrophobic amino acids in each of the sites contribute importantly to the affinity with which cyclic nucleotide is bound.

## Substrate Specificities of PKA and PKG

PKA and PKG-I share many similarities in substrate specificities, and the consensus sequence for phosphorylation by either enzyme is defined as -ArgArgXSer/ThrX-. These kinases can frequently phosphorylate the same proteins, although there are differences. PKG-I phosphorylates numerous proteins in sites that do not conform to this consensus phosphorylation sequence; these sites are not phosphorylated well by PKA. A phenylalanine located C-terminal to the phosphorylation site strongly discriminates against PKA phosphorylation, but not that of PKG, and a basic amino acid adjacent to the phosphorylation site favors PKG-I phosphorylation. The substrate specificity of PKG-II appears to be quite different from those of PKG-I and PKA.

## Negative Feedback and Feed-Forward Control of Cyclic Nucleotide Pathways

A two- to fourfold increase in either cAMP or cGMP typically accounts for the physiological responses to these nucleotides. Negative feedback control mechanisms restrict the magnitude or persistence of cyclic nucleotide

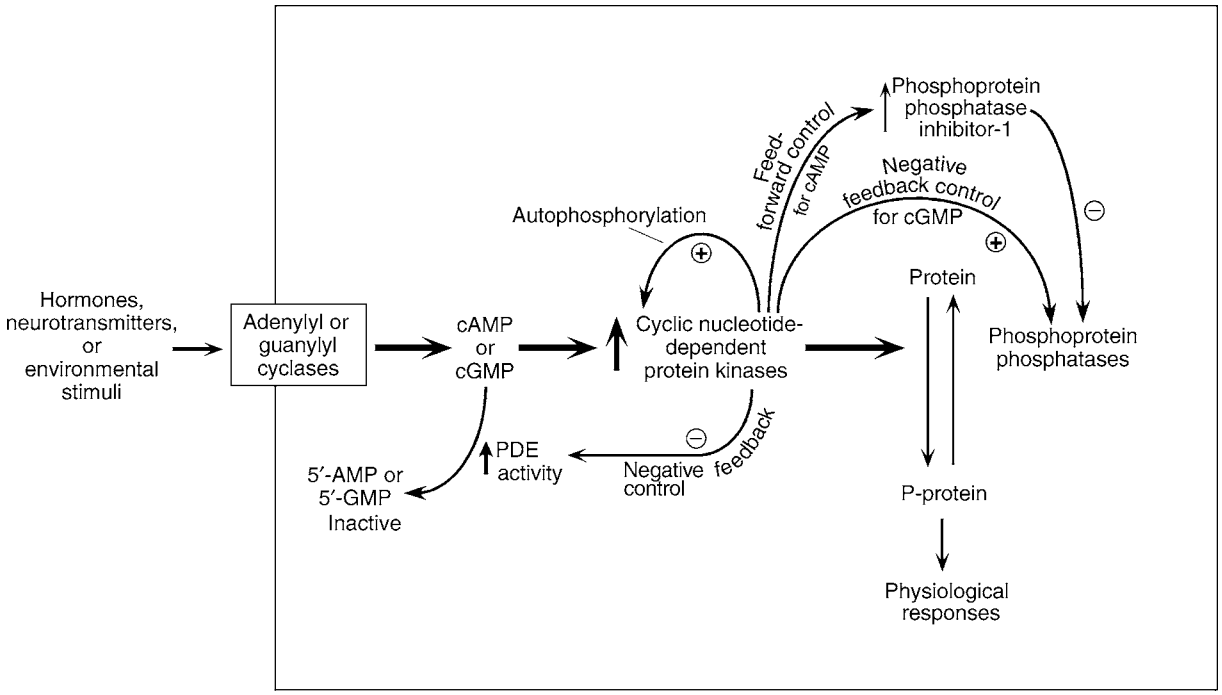



**FIGURE 4** Mechanisms involving phosphorylation that contribute to modulation of the cAMP- and cGMP-signaling pathways. Feedback control refers to processes that tend to alter the overall effectiveness of signaling through cyclic nucleotide pathways. Negative feedback control mechanisms decrease the effectiveness of the cyclic nucleotide signal; these include increased activities of cyclic nucleotide phosphodiesterases (PDE activity), which breakdown the cAMP and cGMP, and increased activities of phosphatases that remove phosphates from phosphoproteins that have been phosphorylated by PKG. Feed-forward control (i.e., positive feedback control) mechanisms enhance the effectiveness of the signal. These include autophosphorylation by PKA and PKGI, which increases the affinity of these enzymes for the respective cyclic nucleotides and increases catalytic activity, and increased phosphorylation of the phosphoprotein phosphatase inhibitor-1 by PKA, which inhibits a phosphoprotein phosphatase and blocks its effect of removing phosphate from phosphoproteins involved in the signaling pathway. Circled + and − denote effects that either increase or decrease as the levels of cyclic nucleotides increase or decrease; arrows, ↑ and ↓, denote a change in the catalytic activity of the indicated enzymes that is induced by PKA or PKG phosphorylation or the state of phosphorylation of the phosphoprotein phosphatase inhibitor-1 in response to increased PKA activity.

signaling whereas feed-forward mechanisms potentiate the response to cyclic nucleotide elevation. Both types of mechanisms have been established for the cAMP and cGMP signaling cascades (Figure 4). PKA and PKG can phosphorylate and activate PDEs, which accelerates the breakdown of cyclic nucleotides, thereby favoring inactivation of the kinases. Both cascades also have feed-forward control because the autophosphorylation of type II PKA and PKG-I increases kinase activity and cyclic nucleotide-binding affinity. The cAMP system has an additional feed-forward control because PKA phosphorylates phosphoprotein phosphatase inhibitor-1 to produce a potent inhibition of phosphoprotein phosphatase action, thereby potentiating the effects of PKA phosphorylation.

## See Also the Following Articles

Adenylyl Cyclases • A-Kinase Anchoring Proteins • Cyclic GMP Phosphodiesterases • Cyclic Nucleotide Phosphodiesterases

## Glossary

**autoinhibition** The effect of one region of an enzyme blocking the action of the catalytic site of another region of that same enzyme.

**autophosphorylation** Protein kinase-catalyzed transfer of the γ-phosphate of ATP to a residue within the protein kinase itself.

**consensus phosphorylation sequence** The amino acid sequence that contains all the determinants that provide for the specific phosphorylation of that sequence by a particular protein kinase.

**heterophosphorylation** Protein kinase-catalyzed transfer of the γ-phosphate of ATP to another protein substrate.

**pseudosubstrate sequence** An amino acid sequence that closely resembles the consensus phosphorylation sequence for a protein kinase substrate but lacks the residue that can be phosphorylated.

## Further Reading

Cummings, D. E., Brandon, E. P., Planas, J. V., Motamed, K., Idzerda, R. L., and McKnight, G. S. (1996). Genetically lean mice result from target disruption of the RII beta subunit of protein kinase A. *Nature* **382**, 622–626.

Eigenthaler, M., Lohmann, S. M., Walter, U., and Pilz, R. B. (1999). Signal transduction by cGMP-dependent protein kinases and their emerging roles in the regulation of cell adhesion and gene expression. *Rev. Physiol. Biochem. Pharmacol.* **135**, 173–209.

Francis, S. H., and Corbin, J. D. (1999). Cyclic nucleotide-dependent protein kinases: Intracellular receptors for cAMP and cGMP action. *Crit. Rev. Clin. Lab. Sci.* **52**, 275–328.

Johnson, D. A., Akamine, P., Radzio-Andzelm, E., Madhusudan, and Taylor, S. S. (2001). Dynamics of cyclic AMP-dependent protein kinase. *Chem. Rev.* **101**, 2243–2270.

Lincoln, T. M., Dey, N., and Sellak, H. (2001). cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J. Appl. Physiol.* **91**, 1421–1430.

Lohmann, S. M., Vaandrager, A. B., Smolenski, A., Walter, U., and De Jonge, H. R. (1997). Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem. Sci.* **22**, 307–312.

Schlossmann, J., Feil, R., and Hofmann, F. (2003). Signaling through NO and cGMP-dependent protein kinases. *Ann. Med.* **35**, 21–27.

Skalhegg, B. S., Huang, Y., Su, T., Idzerda, R. L., McKnight, G. S., and Burton, K. A. (2002). Mutation of the Calpha subunit of PKA leads to growth retardation and sperm dysfunction. *Mol. Endocrinol.* **16**, 630–639.

Smolenski, A., Burkhardt, A. M., Eigenthaler, M., Butt, E., Gambaryan, S., Lohmann, S. M., and Walter, U. (1998). Functional analysis of cGMP-dependent protein kinases I and II as mediators of NO/cGMP effects. *Naunyn Schmiedebergs Arch. Pharmacol.* **358**, 134–139.

## Biography

Dr. Jackie D. Corbin is a Professor in the Department of Molecular Physiology and Biophysics at Vanderbilt University School of Medicine. His career interest has been the mechanism of action of cAMP and cGMP. He has a Ph.D. degree in physiology from Vanderbilt University and did postdoctoral training at the University of California, Davis. He has made seminal discoveries in the mechanisms and functions of cAMP-dependent protein kinases, cGMP-dependent protein kinases, and cyclic GMP-binding cyclic GMP-specific phosphodiesterase (PDE5).

Dr. Sharron H. Francis is a Research Professor in the Department of Molecular Physiology and Biophysics at Vanderbilt University School of Medicine. Her research interest has been the biochemical mechanisms by which cGMP-dependent protein kinases and PDE5 effect and modulate the physiological functions of cGMP. She has a Ph.D. degree in physiology from Vanderbilt University and did postdoctoral training at Washington University and at the National Heart and Lung Institute in Bethesda, MD.

# Cyclic Nucleotide-Regulated Cation Channels

**Martin Biel**
*Ludwig-Maximilians-Universität München, Munich, Germany*

**Franz Hofmann**
*Technischen Universität München, Munich, Germany*

**Cyclic nucleotides exert their physiological effects by binding to four major classes of cellular receptors: cAMP- and cGMP-dependent protein kinases, cyclic GMP-regulated phosphodiesterases, cAMP-binding guanine nucleotide exchange factors, and cyclic nucleotide-regulated cation channels. Cyclic nucleotide-regulated cation channels are unique among these receptors because their activation is directly coupled to the influx of extracellular cations into the cytoplasm and to the depolarization of the plasma membrane. Two families of channels regulated by cyclic nucleotides have been identified, the cyclic nucleotide-gated (CNG) channels and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. The two channel classes differ from each other with regard to their mode of activation. CNG channels are opened by direct binding of cAMP or cGMP. In contrast, HCN channels are principally operated by voltage. These channels open at hyperpolarized membrane potentials and close on depolarization. Apart from their voltage sensitivity, HCN channels are also activated directly by cyclic nucleotides, which act by increasing the channel open probability.**

## General Features of Cyclic Nucleotide-Regulated Cation Channels

Structurally, both CNG and HCN channels are members of the superfamily of voltage-gated cation channels. Like other subunits encoded by this large gene family CNG and HCN channel subunits assemble to tetrameric complexes. The proposed structure and the phylogenetic relationship of mammalian CNG and HCN channel subunits is shown in Figure 1. The transmembrane channel core consists of six $\alpha$-helical segments (S1–S6) and an ion-conducting pore loop between the S5 and S6. The amino- and carboxy-termini are localized in the cytosol. CNG and HCN channels contain a positively charged S4 helix carrying three to nine regularly spaced arginine or lysine residues at every third position. In HCN channels, as in most other members of the channel superfamily, the S4 helix functions as "voltage-sensor" conferring voltage-dependent gating. In CNG channels which are not gated by voltage, the specific role of S4 is not known.

CNG and HCN channels reveal different ion selectivities. CNG channels conduct both $Ca^{2+}$ and monovalent cations with permeability ratios $P_{Ca}/P_{Na}$ ranging from about 2 to 25 depending on the respective channel type and the cyclic nucleotide concentration. By providing an entry pathway for $Ca^{2+}$, CNG channels control a variety of cellular processes that are triggered by this cation. In contrast, HCN channels are not permeable to $Ca^{2+}$. These channels pass $Na^+$ and $K^+$ ion with a relative permeability ratio $P_{Na}/P_K$ of about 0.15–0.25.

In the carboxy-terminus, CNG and HCN channels contain a cyclic nucleotide-binding domain (CNBD) that has significant sequence similarity to the CNBDs of most other types of cyclic nucleotide receptors. In CNG channels, the binding of cyclic nucleotides to the CNBD initiates a sequence of allosteric transitions that lead to the opening of the ion-conducting pore. In HCN channels, the binding of cyclic nucleotides is not required for activation. However, cyclic nucleotides shift the voltage-dependence of channel activation to more positive membrane potentials and thereby facilitate voltage-dependent channel activation. Despite the fact that the CNBDs of HCN and CNG channels show significant sequence homology, the two channel classes reveal different selectivities for cyclic nucleotides. HCN channels display an approximately 10-fold higher affinity for cAMP than for cGMP, whereas CNG channels select cGMP over cAMP.

## CNG Channels

CNG channels are expressed in retinal photoreceptors and olfactory neurons and they play a key role in

 © 2004, Elsevier Inc. All Rights Reserved. 

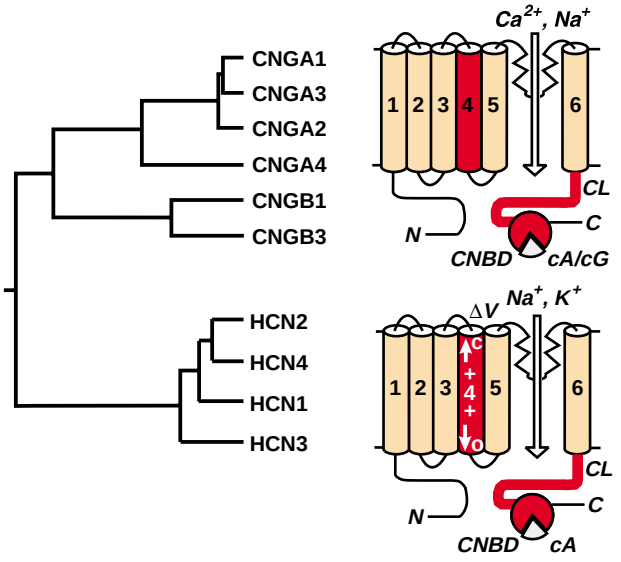



**FIGURE 1** Phylogenetic tree and structural model of cyclic nucleotide-regulated cation channels. The CNG channel family comprises six members, which are classified into A subunits (CNGA1–4) and B subunits (CNGB1 and CNGB3). The HCN channel family comprises four members (HCN1–4). CNG and HCN channels share a common transmembrane topology, consisting of six transmembrane segments (1–6), a pore loop, and a cyclic nucleotide-binding domain (CNBD). CNG channels are activated *in vivo* by binding of either cAMP (cA) or cGMP (cG), depending on the channel type. HCN channels activate on membrane hyperpolarization (ΔV) and are enhanced by binding of cAMP. Structures involved in channel gating are shown in red. The positively charged amino acid residues in the S4 segment of HCN channels are indicated by (+). The movement of the S4 leading to channel closure (c) or opening (o) is indicated by arrows. CL denotes the C-linker involved in activation gating of CNG and HCN channels.

visual and olfactory signal transduction. CNG channels are also found at low density in some other cell types and tissues such as brain, testis, and kidney. While the function of CNG channels in sensory neurons has been unequivocally demonstrated, the role of these channels in other cell types remains to be established. Based on phylogenetic relationship, the six CNG channels identified in mammals are divided in two subfamilies, the A subunits (CNGA1-4) and the B subunits (CNGB1 and CNGB3) (Figure 1). CNG channel A subunits (with the exception of CNGA4) can form functional homomeric channels in various heterologous expression systems. In contrast, B subunits do not give rise to functional channels when expressed alone. However, together with CNGA1–3 they confer novel properties (e.g., single-channel flickering, increased sensitivity for cAMP and L-cis diltiazem) that are characteristic of native CNG channels. The physiological role and subunit composition is known for three native CNG channels: the rod photoreceptor channel, the cone photoreceptor channel, and the olfactory channel. The CNG channel of rod photoreceptors is a tetramer composed of three CNGA1 and one long isoform of the CNGB1 subunit (CNGB1a). The cone photoreceptor channel consists of the CNGA3 and the CNGB3 subunit. CNG channels control the membrane potential and the calcium concentration of photoreceptors. In the dark, both channels are maintained in the open state by a high concentration of cGMP. The resulting influx of $Na^+$ and $Ca^{2+}$ (dark current) depolarizes the photoreceptor and promotes synaptic transmission. Light-induced hydrolysis of cGMP leads to the closure of the CNG channel. As a result, the photoreceptor hyperpolarizes and shuts off synaptic glutamate release. Mutations in CNG channel genes have been linked to retinal diseases. Mutations in the CNGA and CNGB1 subunits have been identified in the genome of patients suffering from retinitis pigmentosa. The functional loss of either the CNGA3 or the CNGB3 subunit causes total color blindness (achromatopsia) and degeneration of cone photoreceptors.

The CNG channel expressed in cilia of olfactory neurons consists of three different subunits: CNGA2, CNGA4, and a short isoform of the CNGB1 subunit (CNGB1b). The channel is activated *in vivo* by cAMP that is synthesized in response to the binding of odorants to their cognate receptors. The olfactory CNG channel is thought to conduct almost exclusively $Ca^{2+}$ under physiological ionic conditions. The resulting increase in cellular $Ca^{2+}$ activates a $Ca^{2+}$-activated $Cl^-$ channel that further depolarizes the cell membrane. $Ca^{2+}$ is not only a permeating ion of the olfactory CNG channel, it also represents an important modulator of this channel. By forming a complex with calmodulin, which binds to the CNGA2 subunit, $Ca^{2+}$ decreases sensitivity of the CNG channel to cAMP.

The resulting inhibition of channel activity is the principal mechanism underlying odorant adaptation. Recent studies indicate that CNGA4 and the CNGB1b units also contribute to the CaM-dependent feedback inhibition.

## HCN Channels

A cation current that is slowly activated by membrane hyperpolarization (termed $I_h$, $I_f$, or $I_q$) is found in a variety of excitable cells including neurons, cardiac pacemaker cells, and photoreceptors. The best understood function of $I_h$ is the control of heart rate and rhythm by acting as "pacemaker current" in the sinoatrial (SA) node. $I_h$ is activated during the membrane hyperpolarization following the termination of an action potential and provides an inward $Na^+$ current that slowly depolarizes the plasma membrane. Sympathetic stimulation of SA node cells raises cAMP levels and increases $I_h$ by a positive shift of the current activation curve, thus accelerating diastolic depolarization and heart rate. Stimulation of muscarinic receptors slows

down heart rate by the opposite action. In the brain, $I_h$ fulfills diverse functions: it controls the activity of spontaneously spiking neurons (neuronal pacemaking), it is involved in the determination of resting potential, it provides rebound depolarizations in photoreceptors in response to pronounced hyperpolarizations, it is involved in the transduction of sour taste and it is involved in the control of synaptic plasticity.

HCN channels represent the molecular correlate of the $I_h$ current. In mammals, the HCN channel family comprises four members (HCN1–4) that share about 60% sequence identity to each other and about 25% sequence identity to CNG channels. The highest degree of sequence homology between HCN and CNG channels is found in the CNBD. When expressed in heterologous systems, all four HCN channels generate currents displaying the typical features of native $I_h$: (1) activation by membrane hyperpolarization, (2) permeation of $Na^+$ and $K^+$, (3) positive shift of the voltage-dependence of channel activation by direct binding of cAMP, (4) channel blockade by extracellular $Cs^+$. HCN1–4 mainly differ from each other with regard to their speed of activation and the extent by which they are modulated by cAMP. HCN1 is the fastest channel, followed by HCN2, HCN3, and HCN4. Unlike HCN2 and HCN4 whose activation curves are shifted by about + 15 mV by cAMP, HCN1 is only weakly affected by cAMP (shift of less than +5 mV). Site-directed mutagenesis experiments have provided insight into the complex mechanism underlying dual HCN channel activation by voltage and cAMP. Surprisingly, the voltage-dependent movement of the positively charged S4 helix is fully conserved between HCN channels and depolarization-activated channels such as Shaker K-channels. However, the allosteric coupling between S4 movement and the activation gate is different in the two channel types. In HCN channels, inward movement of S4 leads to the opening of the channel gate, whereas it closes Shaker channels. Major determinants affecting channel activation are the intracellular S4–S5 loop, the S5–P linker, the S1 segment, and the extracellular S1–S2 loop. The CNBD fulfills the role of an auto-inhibitory channel domain. In the absence of cAMP, the cytoplasmic carboxy-terminus inhibits HCN channel gating by interacting with the channel core and thereby shifts the activation curve to more hyperpolarizing voltages. Binding of cAMP to the CNBD relieves this inhibition. Differences in the magnitude of the response to cAMP among the four HCN channel isoforms are largely due to differences in the exent to which the CNBD inhibits basal gating. It remains to be determined if the inhibitory effect of the CNBD is conferred by a direct physical interaction with the channel core domain or by some indirect pathway. There is initial evidence that the so-called C-linker, a peptide of about 80 amino acids that connects the last transmembrane helix (S6) to the CNBD, plays an important role in this process (Figure 1). The C-linker was also shown to play a key role in the gating of CNG channels, suggesting that the functional role of this domain has been conserved during channel evolution.

Genetic deletion experiments in mice have been performed to facilitate the analysis of the physiological and pathophysiological role of individual HCN channel subunits in neuronal and cardiac function. Mice lacking the HCN2-subunit are viable but display a complex neurological–cardiological phenotype including absence epilepsy, ataxia, and sinus arrhythmia. As a likely explanation for this phenotype it was found that the loss of HCN2 induces a pronounced shift of the resting membrane potential and thereby impairs regular cardiac and neuronal rhythmicity. HCN4 represents the predominantly expressed HCN channel isoform in SA node. Deletion of HCN4 results in embryonic lethality after day 9.5 dpc. Electrophysiological studies with HCN4-deficient embryonic heart cells revealed that HCN4 underlies most if not all of the cardiac $I_f$ current at early embryonic stages. Furthermore, HCN4 seems to be required for normal differentiation of the cardiac pacemaker system. Mice lacking the brain-specific HCN1 show a motor deficit, which is likely a result of altered firing rhythms in Purkinje neurons.

## See Also the Following Articles

Ligand-Operated Membrane Channels: Calcium (Glutamate) • Ligand-Operated Membrane Channels: GABA • Olfactory Receptors • Photoreceptors

## Glossary

**channel states** Voltage-gated channels have at least three states: a closed, an open, and an inactivated state. Ions conduct through the channel at the open state.

**ligand-gated channel** In contrast to voltage-gated channels, ligand-gated channels are opened by the occupation of the ligand binding site. HCN channels are gated by the membrane potential and are modulated by the binding of cAMP.

**olfaction** The processing of smell in the nose and olfactory bulb.

**pacemaker** Specialized cells that depolarize at a known frequency. The best-known example is the sino-atrial node of the heart.

**photoreceptor** Part of the rod or cone in the retina that senses light.

## Further Reading

Biel, M., Seeliger, M., Pfeifer, A., Kohler, K., Gerstner, A., Ludwig, A., Jaissle, G., Fauser, S., Zrenner, E., and Hofmann, F. (1999). Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3. *Proc. Natl Acad. Sci. USA* **96**, 7553–7557.

Biel, M., Schneider, A., and Wahl, C. (2002). Cardiac HCN channels: Strucuture, function and modulation. *Trends Cardiovasc. Med.* **12**, 206–213.

Dzeja, C., Hagen, V., Kaupp, U. B., and Frings, S. (1999). $Ca^{2+}$ permeation in cyclic nucleotide-gated channels. *EMBO. J.* **18**, 131–144.

Kaupp, U. B., and Seifert, R. (2002). Cyclic nucleotide-gated ion channels. *Physiol. Rev.* **82**, 769–824.

Ludwig, A., Budde, T., Stieber, J., Moosmang, S., Wahl, C., Holthoff, K., Langebartels, A., Wotjak, C., Munsch, T., Zong, X., Feil, S., Feil, R., Lancel, M., Chien, K. R., Konnerth, A., Pape, H. C., Biel, M., and Hofmann, F. (2003). Absence epilepsy and sinus dysrhythmia in mice lacking the pacemaker channel HCN2. *EMBO J.* **15**, 216–224.

Männikkö, R., Elinder, F., and Larsson, H. P. (2002). Voltage-sensing mechanism is conserved among ion channels gated by opposite voltages. *Nature* **419**, 837–841.

Munger, S. D., Lane, A. P., Zhong, H., Leinders-Zufall, T., Yau, K. Y., Zufall, F., and Reed, R. R. (2001). Central role of the CNGA4 channel subunit in $Ca^{2+}$-calmodulin-dependent odor adaptation. *Science* **294**, 2172–2175.

Robinson, R. B., and Siegelbaum, S. A. (2003). Hyperpolarization-activated cation currents: From molecules to physiological function. *Ann. Rev. Physiol.* **63**, 453–480.

Wainger, B. J., DeGennaro, M., Santoro, B., Siegelbaum, S. A., and Tibbs, G. R. (2001). Molecular mechanism of cAMP modulation of HCN pacemaker channels. *Nature* **411**, 805–810.

Zhong, H., Molday, L. L., Molday, R. S., and Yau, K. W. (2002). The heteromeric cyclic nucleotide-gated channel adopts a 3A:1B stoichiometry. *Nature* **420**, 193–198.

## Biography

Martin Biel is a Professor in Pharmacology and Chair of the Institute of Pharmakologie für Naturwissenschaftler, Science Faculty at the Ludwigs-Maximillian-University München. His research interests are cyclic nucleotide regulated ion channels and CNG and HCN channels. He holds a Ph.D. in Pharmacy. He obtained his Habilitation in Pharmacology at the Medical Faculty of the Technische Universität München.

Franz Hofmann is Professor in Pharmacology and Chair of the Institute for Pharmakologie und Toxikologie, Medical Faculty at the Technische Universität München. His primary research interest is cyclic nucleotide-regulated cellular functions, including cGMP protein kinases and ion channels. He holds an M.D. from the Universität Heidelberg. His group has contributed many seminal papers on the regulation of L-type calcium channels, CNG-channels, pacemaker channels, and the biology of cGMP kinase.

# Cysteine Proteases

**David J. Buttle**
*University of Sheffield, Sheffield, UK*

**John S. Mort**
*Shriners Hospital for Children, Montréal, Canada*

**Cysteine proteases represent one of the four main groups of peptide-bond hydrolases. They all use a $S^-$ anion of a cysteine side chain as the nucleophile in peptide-bond hydrolysis. Cysteine proteases are found in all forms of life and mediate a wide variety of physiological and pathological processes, from the bulk digestion of protein, on the one hand, to highly regulated rate-determining and specific peptide-bond cleavages at the opposite extreme. They are implicated in a number of human pathologies, and the development of cysteine protease inhibitors as drugs is a very active field.**

## Phylogenetic Relationships

The use of the cysteine residue thiolate ion as the nucleophile for peptide-bond cleavage appears to have been derived at least five times during evolution using different structural frameworks, and members of each of these protease families are widely distributed. However, the bulk of these enzymes are members of clans CA and CD as classified in the MEROPS database (a regularly updated and invaluable source of information on proteases) (Figure 1). Clan CA contains the earliest identified cysteine proteases, such as the papaya protease papain, and the mammalian proteases, for example cathepsins B, L, S, and K. These enzymes are generally present in lysosomes or in other membrane-bound compartments where they mediate antigen processing and presentation, or they are secreted from the cell. As a group, clan CA proteases show cleavage selectivity for the residue 2 positions N-terminal to the cleavage site. A related subfamily of papain-like enzymes is the calcium-dependent calpains, which are found in the cytoplasm and mediate various intracellular processes.

The second major group of cysteine proteases, clan CD, has been described much more recently and includes the caspases, legumains, several viral proteases, and separase, which is responsible for the separation of sister chromatids during mitosis. Unlike the papain family, these proteases generally show exquisite specificity for cleavage C-terminal to particular amino acid residues – aspartic acid in the case of caspases, asparagine for legumains, and arginine for separase. The caspases are the mediators of apoptosis.

## Mechanism

Proteolytic enzymes are very efficient catalysts that can increase the rate of peptide-bond hydrolysis a billion-fold. In the case of the cysteine proteases, the catalytic mechanism has been most extensively investigated for the clan CA enzymes. Despite their distinct evolutionary origins, it appears that the mechanism of other cysteine proteases is quite similar (an example of convergent evolution). Cleavage of the peptide bond is dependent on a thiolate-imidazolium ion pair provided by the cysteine and histidine residues in the active site (Figure 2). The environment of the active site results in an unusually low $pK_a$ of around 4 for the cysteine thiol group. Nucleophilic attack on the carbonyl group results in the formation of a tetrahedral oxyanion intermediate. This then accepts a proton from the imidazolium group, resulting in the formation of an acyl enzyme intermediate and the release of the C-terminal portion of the substrate. In a second reaction, the acyl enzyme intermediate is deacylated by a water molecule and the remaining portion of the substrate is released.

## Control

### Activation

As is the case with most proteolytic enzymes, cysteine proteases are synthesized as inactive precursors, allowing them to be delivered to their intended site of action without harming the biosynthetic machinery of the cell. Often these proenzymes are retained in an inactive form until their services are required. Very different strategies have evolved for the activation of the precursors of the different cysteine protease groups. In the case of the

 © 2004, Elsevier Inc. All Rights Reserved. 

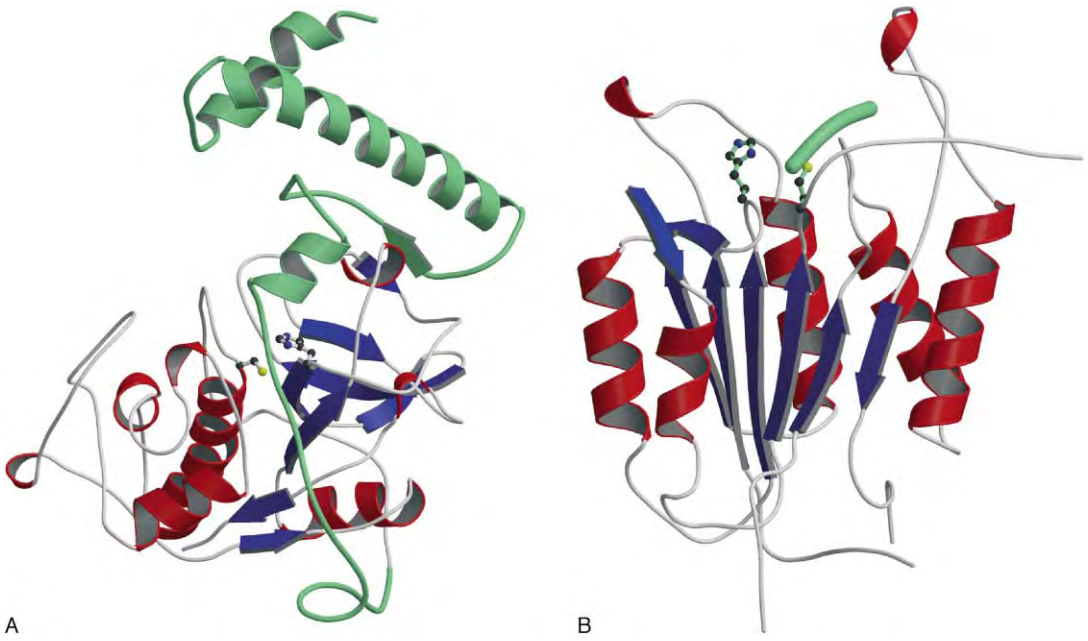



**FIGURE 1** Cysteine protease structure. Ribbon diagrams of representative members of the two principal cysteine peptidase clans: (A) cathepsin K (pdb 1by8), a member of the papain family in Clan CA shown as the proenzyme, and (B) caspase-3 (pdb 1cp3), a member of clan CD. The active site cysteine and histidine residues are shown in ball and stick representation. For caspase-3, the position occupied by the substrate is indicated by the green rod. For procathepsin K, the proregion is depicted in green. The extended coil linking the helical region of the propiece passes through the active site in the region normally occupied by the substrate. Processing of the proforms of papain family members requires the proteolytic removal of the proregion without any change to the structure of the protease component. In contrast, major restructuring of the active site occurs during caspase activation and the active form of the enzyme is a dimer of the structure shown.

papain family, an N-terminal propeptide blocks access to the fully functional active site by binding in the reverse orientation to that required for peptide-bond cleavage (Figure 1). Its removal via inter- or intramolecular proteolysis liberates the functional protease. In contrast, the caspases undergo dimerization, which signals a rearrangement of the protein to form the previously incomplete active site.

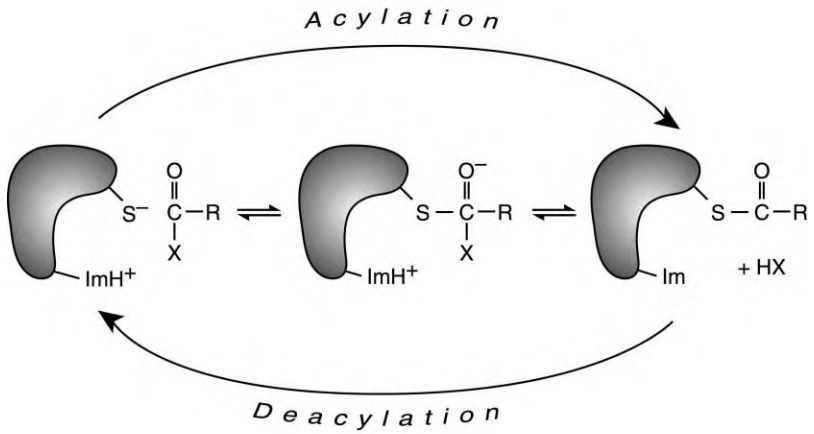



**FIGURE 2** Cysteine protease mechanism. Scheme for the reaction mechanism of cysteine protease action. The active-site cysteine and histidine residues are present as a thiolate ($S^-$)-imidazolium ($ImH^+$) ion pair. The substrate is represented by the carbonyl group under attack connected to the N-terminal peptide component R and the C-terminal component X. Upon substrate binding, nucleophilic attack by the thiolate ion results in the formation of a tetrahedral oxyanion intermediate. This, following proton donation from $ImH^+$, breaks down to form an acyl enzyme (thiol ester) intermediate with the release of the C-terminal fragment HX. Adapted from Polgár and Asbóth (1986). The basic difference in catalysis by serine and cysteine proteinases resides in charge stabilization in the transition state. *J. Theor. Biol.* **121**, 323–326.

### Inhibitors

A further level of control for unwanted proteolytic activity is provided by the action of a series of cysteine protease inhibitors. Members of the cystatin family are highly effective inhibitors of papain-like enzymes. The N terminus of the cystatin molecule binds in the active site cleft in a substrate-like manner, but the inhibitor evades cleavage by the target protease due to the presence of a glycine residue at what would normally be the site of cleavage. Additional elements in the inhibitor bind to the protease to maneuver the N-terminal substrate region just out of the range of the catalytic residues. A separate family of inhibitors, termed inhibitors of apoptosis proteins (IAPs), is available for the caspases. These proteins bind across the active caspase, obstructing normal substrate access but making limited interactions with the protease. Analogous to the propeptides of the papain family members, IAPs ensure their resistance to proteolysis by binding in the reverse direction to that required for substrate cleavage.

## Physiology

### Lysosomal Proteolysis

Virtually all eukaryotic cells possess membrane-enclosed organelles known as lysosomes This structure is the site of breakdown of endocytosed or phagocytosed material, and it is also the location for the turnover of certain cytosolic proteins and organelles. In most eukaryotic cells, the lysosome has the highest concentration of cysteine proteases and other hydrolases, which implies that these enzymes are involved in protein degradation for the recycling of amino acids for protein synthesis. This is indeed likely to be the major physiological function of ubiquitously expressed cysteine proteases such as cathepsin L, legumain, cathepsin B, and cathepsin H. Indeed, the last two enzymes, which fragment polypeptides (endopeptidase action) and then attack the fragments at either the C-terminal end, in the case of cathepsin B (carboxypeptidase action), or at the amino terminus, in the case of cathepsin H (aminopeptidase action), are functionally well equipped to fulfill a digestive role.

### Antigen-Ii Processing

In the major histocompatibility complex (MHC) class II antigen-presenting cells, such as dendritic cells, macrophages, and B lymphocytes, the lysosomal or endocytic localization of some cysteine proteases has allowed them to participate in antigen presentation to T cells. In this process, the antigen is endocytosed and fragmented into peptides of between 13 and 26 amino acids in length. In order to allow the peptides thus generated to bind to the MHC complexes for presentation at the cell surface, the invariant chain Ii must be cleaved in order to remove it from the peptide-binding groove. Cathepsin S has been known for some time to be highly expressed in antigen-presenting cells and recently the use of selective cathepsin S inhibitors and the generation of cathepsin S-null mice has demonstrated an important, but not solitary, role for this enzyme in Ii processing.

It is likely that a number of endosomal proteases are involved in antigen processing and also that the protease involved is dependent on the nature of the antigen. In some cases at least, legumain appears to play an important role.

### Bone Turnover

Bone is continually being broken down and replenished. This is important in calcium homeostasis, in which soluble calcium is provided by the hydrolysis of the mineral fraction of bone. At the same time, the organic component of bone, consisting largely of type I collagen, is also removed. Although a normal physiological process, under some circumstances bone resorption can outstrip synthesis. This can lead to the condition known as osteoporosis or brittle bone disease, which largely affects postmenopausal women. Other situations in which bone resorption is outside normal homeostatic control include bone metastases and some rare genetic diseases. The process of bone resorption is mediated largely by the multinucleated cell type known as an osteoclast. During periods of active resorption, osteoclasts sitting on bone surfaces generate a ruffled membrane, effectively sealing off the area immediately beneath the cell into which proteolytic enzymes and acid are secreted to remove the organic and mineral components, respectively. Both cysteine proteases and matrix metalloproteinases have been implicated in the hydrolysis of the organic component of bone, and it is likely that the relative contributions played by these different enzymes depend on the type of bone, with cysteine proteases playing a dominant role in the resorption of long bone.

The discovery that pycnodysostosis, an autosomal recessive disease leading to short stature and bone fragility, was due to a deficiency in cathepsin K activity, provided the first indication that this cysteine protease played a major role in bone resorption. Laboratory experiments have demonstrated this enzyme's efficiency at cleaving type I collagen, and consequently it has become a major drug target for the treatment of bone-resorbing diseases.

Other cysteine proteases are likely to be involved in bone resorption. For instance, legumain acts as an inhibitor of bone resorption by inhibiting the formation of multinucleated osteoclasts from their mononuclear precursors. The precise mechanism is not known, but it is not linked to the catalytic activity of the enzyme, and

in fact the active component is the C-terminal peptide that is removed upon generation of full catalytic activity.

### Thyroglobulin Processing

The distribution of cathepsin K is quite restricted, being found predominantly in the ovary and in the osteoclasts, the bone-degrading cells. The other site where cathepsin K is highly expressed, alongside the more ubiquitously distributed cathepsins B and L, is in the thyroid follicles, which are the site of storage of thyroglobulin, the precursor of the thyroid hormones. It appears from experiments using single- and double-knockout mice that all three of these cysteine proteases are involved in thyroid hormone generation from thyroglobulin. All are found in the lumen of thyroid follicles, where cathepsin L appears to be involved in both the solubilization of cross-linked thyroglobulin and subsequent generation of thyroid hormones. In contrast, cathepsin B may be primarily involved in thyroglobulin solubilization and cathepsin K in thyroid hormone generation.

### Apoptosis

The importance of programmed cell death to developmental processes has been recognized for many years. The discovery that the proapoptotic *ced-3* gene of *Caenorhabditis elegans* encodes a protease homologous to the mammalian cysteine protease interleukin-1$\beta$-converting enzyme (ICE), together with the demonstration that ICE induces apoptosis in mammals, ignited a great deal of renewed interest in the field, due in large part to the desire of the pharmaceutical industry to control the process. This article is too short to do justice to such a large area of research, which has been the subject of a number of good reviews (see Further Reading). Briefly, the proteases most intricately involved in the process of apoptosis are the caspases (including ICE), all of which have acute specificity for the cleavage of aspartyl bonds and which function in a cascade reaction. Upstream caspases activate downstream caspases, which cleave proteins whose functions are essential to cell survival. One of the initiators of this cascade event is the occupation of cell surface death receptors of the tumor necrosis factor receptor 1 family, which triggers the activation of caspase 8. Alternatively, the formation of a complex with cytochrome c and Apaf-1 in response to DNA damage leads to the activation of caspase 9. Both of these initiating caspases then activate the downstream executioner caspases, such as caspase 3. In cell killing by cytotoxic T cells or NK cells, this pathway is highjacked in the target cell through the ability of the killing cell's serine proteinase, granzyme B, to activate caspases.

It has become increasingly clear that, under some circumstances, cysteine proteases other than caspases are involved in apoptosis. For instance, cathepsin B may be involved in death receptor-mediated apoptosis of tumor cells if downstream caspases are inhibited. This process is expedited by tumor necrosis factor- and caspase 8-induced increases in cytosolic cathepsin B and subsequent release from mitochondria of cytochrome c.

## Pathology

Cysteine proteases have been implicated in a wide range of pathologies, including cancer, osteoporosis, and arthritis. Here we concentrate on their involvement in two other pathological situations that may be less fully appreciated.

### Allergenic Proteinases

Proteases represent a disproportionately high number of allergens. Examples include the major allergen from the house dust mite (a cysteine protease that is the product of the *Der p*1 gene) and many plant cysteine proteases such as actinidain, bromelain, papain, and glycyl endopeptidase. The normal route for the generation of atopy involves the initiation of a Th2 lymphocyte phenotype and enhanced IgE responses by B cells. Although the mechanisms by which cysteine (and other) proteases favor a switch to a Th2-like response are not completely elucidated, a link with the catalytic activity of these enzymes has been made. The mechanisms may be many and varied and include the cleavage (and activation?) of IgE and IL-2 receptors and disruption of epithelial tight junctions, thus enhancing access by allergens to immune cells. However, a systematic analysis of potential molecular targets for these enzymes has not been undertaken.

### Parasitic Proteinases

Many parasites contain cysteine proteases in their phagocytic vacuoles, where they are involved in the digestion of engulfed material to be used in satisfying the nutritional requirements of the organism. As such, these enzymes are potential therapeutic targets. Examples include *Entamoeba histolytica*, a causative agent of amoebic dysentery; trypanosomatids that cause African sleeping sickness and Chagas' disease; schistosomal organisms and the plasmodial parasites that are the causative agents of malaria; and a number of parasitic helminths. In the case of plasmodial parasites, the cysteine proteases are known to be instrumental in the degradation of host hemoglobin, the principal source of amino acids for the parasite, because enzyme inhibition blocks hemoglobin degradation and parasite development. In a mouse model of the human disease, an orally administered inhibitor of the enzyme produced a 40%

cure rate, and a delayed progression of the disease in the remaining animals. New treatments for this and other parasitic diseases that kill millions every year are badly needed, due to increasing resistance to currently available drugs.

## See Also the Following Articles

Calpain • Caspases and Cell Death • Cell Death by Apoptosis and Necrosis • Metalloproteases • SUMO Modification

## Glossary

**endocytosis** The taking in by a cell of soluble or membrane-bound material from outside the cell. In the case of eukaryotic cells, endocytosed material passes into the endosome, a membrane-limited organelle that can fuse with lysosomes to allow digestion of the contents.

**homeostasis** The normal balance of a healthy living organism, in which the status quo is maintained by regulatory mechanisms such as feedback control. In protein turnover, it is the balance between protein synthesis and protein breakdown.

**hydrolase** An enzyme that breaks peptide, ester, or glycoside bonds by the addition of water. In the case of proteases, water is split, and the resulting oxygen is added to the newly formed carboxyl terminus while the two hydrogens are added to the new amino terminus formed by the breaking of the peptide bond.

**protease** An enzyme that cleaves proteins or peptides (also called proteinase, peptidase, or proteolytic enzyme).

**specificity** Susceptibility of an amino acid sequence of a peptide or protein to cleavage by a particular protease. Most proteases possess specificity for the cleavage of particular sequences. For instance, caspases cleave immediately after aspartic acid residues (they cleave aspartyl bonds); most lysosomal cysteine proteases cleave the second peptide bond on the carboxyl side of a hydrophobic residue, the exception being legumain, which cleaves asparaginyl bonds. Specificity is conferred by the structure of the substrate-binding groove on the protease.

## Further Reading

Barrett, A. J., Rawlings, N. D., and Woessner, J. F. (eds.) (1998). *Handbook of Proteolytic Enzymes*. Academic Press, London.

Chapman, H. A., Riese, R. J., and Shi, G.-P. (1997). Emerging roles for cysteine proteases in human biology. *Annu. Rev. Physiol.* **59**, 63–88.

Cygler, M., and Mort, J. S. (1997). Proregion structure of members of the papain superfamily: Mode of inhibition of enzymatic activity. *Biochimie* **79**, 645–652.

Delaissé, J.-M., Engsig, M. T., Everts, V., del Carmen Ovejero, M., Ferreras, M., Lund, L., Vu, T. H., Werb, Z., Winding, B., Lochter, A., Karsdal, M. A., Troen, T., Kirkegaard, T., Lenhard, T., Heegaard, A.-M., Neff, L., Baron, R., and Foged, N. T. (2000). Proteinases in bone resorption: Obvious and less obvious roles. *Clin. Chim. Acta* **291**, 223–234.

Elliott, E., and Sloane, B. F. (1996). The cysteine protease cathepsin B in cancer. *Perspect. Drug Discovery Design* **6**, 12–32.

Friedrichs, B., Tepel, C., Reinheckel, T., Deussing, J., von Figura, K., Herzog, V., Peters, C., Saftig, P., and Brix, K. (2003). Thyroid functions of mouse cathepsins B, K, and L. *J. Clin. Invest.* **111**, 1733–1745.

Lang, A., Hörler, D., and Baici, A. (2000). The relative importance of cysteine peptidases in osteoarthritis. *J. Rheumatol.* **27**, 1970–1979.

MEROPS database. Available at: http://merops.sanger.ac.uk.

Polgár, L., and Asbóth, B. (1986). The basic difference in catalysis by serine and cysteine proteinases resides in charge stabilization in the transition state. *J. Theor. Biol.* **121**, 323–326.

Shakib, F., Schulz, O., and Sewell, H. (1998). A mite subversive: Cleavage of CD23 and CD25 by Der p1 enhances allergenicity. *Immunol. Today* **19**, 313–316.

Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: Enemies within. *Science* **281**, 1312–1316.

## Biography

David J. Buttle is Reader in Matrix Biology in the Division of Genomic Medicine at the University of Sheffield, U.K. His principal research interests are the turnover of extracellular matrix proteins by proteases and how this relates to various diseases such as arthritis, multiple sclerosis, and cancer. He received a Ph.D. from the Council for National Academic Awards, U.K., before receiving postdoctoral training at the Strangeways Research Laboratory, Cambridge, U.K.

John S. Mort is a Principal Investigator at the Shriners Hospital for Children and an Associate Professor in the Departments of Surgery and Medicine at McGill University in Montreal, Canada. His research program aims to determine the role of specific proteolytic enzymes in connective tissue remodeling during growth and development and in its destruction in arthritis. He received his Ph.D. from McMaster University, Hamilton, Canada, and his postdoctoral training at the University of Western Ontario.

# Cytochrome $b_6f$ Complex

**Günter A. Hauska and Thomas Schödl**
*Universität Regensburg, Regensburg, Germany*

**The cytochrome $b_6f$-complex connects oxygen evolution by photosystem II, with NADPH production by photosystem I (PSI), in the photosynthetic electron transport (ET) chain of plants, algae, and cyanobacteria. More specifically, it reoxidizes plastoquinol, which has been produced in the membrane by PSII with electrons coming from water, at the expense of plastocyanin or cyt *c*, the electron donors to PSI. It belongs to the family of the cytochrome *bc*-complexes, which function at central position in many eukaryotic and prokaryotic ET chains. They all oxidize quinol in a proton translocating redox reaction, which is best described by the so-called Q-cycle. The resulting transmembrane electrochemical proton gradient drives ATP synthesis in a rotatory mechanism of the $H^+$-translocating F1Fo-ATP synthases. The scenario holds for respiration and photosynthesis, from higher organisms down to bacteria and archaea.**

**Quinol oxidation is the rate-limiting step in these ET chains ($\tau \sim$ ms), and therefore a key point for regulation. Plastoquinol oxidation by the cyt $b_6f$-complex in oxygenic photosynthesis, for example, controls the distribution of light quanta to the two photosystems, via a protein kinase, which by phosphorylation determines the association of chlorophyll antenna proteins with the reaction centers.**

**Crystal structures of the cytochrome $b_6f$-complex at atomic resolution have recently been obtained for the prokaryotic cyanobacterium *Mastigogladus laminosus* as well as for the eukaryotic green alga *Chlamydomonas reinhardtii*. They largely resemble the dimeric structures for the cytochrome $bc_1$-complex from mitochondria, but also reveal some striking differences. In addition to the four redox centers – two hemes *b*, one heme *c*, and a high potential 2Fe2S-cluster per monomeric complex – which are shared with the cyt $bc_1$-complex, the cyt $b_6f$-complex per monomer contains one chlorophyll *a*, one carotenoid, and most surprisingly, an extra heme.**

## Discovery and Isolation

Cytochromes $b_6$ and $f$ were discovered in leaves by Robin Hill in the middle of last century.

The reduction of cyt $f$ by red light absorbed in PSII and its reoxidation by far-red light absorbed by PSI led to the formulation of the "Z-scheme" of photosynthetic ET in chloroplasts, with the two light reactions acting in series, connected by cyt $f$.

The first evidence for a complex between these two cytochromes was provided by Nelson and Neumann (Tel-Aviv) in 1972, who recognized that a particle isolated from chloroplasts contained redox centers similar to the cyt $bc_1$-complex from mitochondria – heme $b$, heme $c$, and nonheme iron. A little later Wood and Bendall described a plastoquinol–plastocyanin oxidoreductase activity solubilized from chloroplasts. Both observations were combined by Hurt and Hauska, who succeeded in the isolation of the complex with a well-defined composition in functionally active form from spinach chloroplasts. In addition to plastoquinol–plastocyanin oxidoreductase activity, the isolate catalyzed oxidant-induced reduction of cyt $b_6$, a signature of the Q-cycle mechanism. Furthermore, during the redox reaction it transcolated protons across the membrane in an electrogenic way, after incorporation into lipid vesicles.

The isolation procedure involved four steps – removal of peripheral proteins, selective solubilization by a mild, nonionic detergent, ammonium sulfate precipitation, and sucrose density gradient centrifugation. These procedures have been modified repeatedly, resulting in improved isolates with turnover numbers up to $500\ s^{-1}$, from spinach as well as from other organisms, but in principle the strategy is followed until today.

## Composition and Structure

The cyt $b_6f$-complex consists of four major polypeptides with two pairs of redox centers which differ by 300–400 mV in the redox potentials under standard conditions. The pair with the higher redox potentials is represented by a heme $c$ (+340 mV) covalently bound to cyt $f$, and by the 2Fe2S-cluster (+300 mV) of the Rieske FeS–protein. The pair with the lower potentials are the two hemes $b$ on cyt $b_6$ (−50 and −150 mV). The fourth polypeptide (subunit IV) lacks a redox center. According to a nomenclature for cyanobacteria, cyt $f$, cyt $b_6$, the Rieske FeS–protein, and subunit IV are also known as

 © 2004, Elsevier Inc. All Rights Reserved. 

PetA, PetB, PetC, and PetD, respectively (Pet standing for photosynthetic electron transport), and are coded by the corresponding *pet* genes.

The folding of these subunits in the thylakoid membrane is depicted in Figure 2. They correspond to the central components in the cyt $bc_1$-complex of mitochondria and bacteria. Cyt $c_1$ (~33 kDa) resembles cyt *f*, the Rieske FeS–protein (some 20 kDa) occurs in both systems, and the N-terminal part of cyt *b* (~42 kDa) is equivalent to cyt $b_6$ (24 kDa), while its C-terminal part corresponds to subunit IV (17 kDa).

Several bacterial cyt $bc_1$-complexes contain just these central components, cyt $c_1$, cyt *b* and the Rieske FeS–protein, while the eukaryotic $bc_1$-complexes in mitochondria contain 7–8 "supernumerary" subunits, which do not occur in the cyt $b_6f$-complex. On the other hand four small hydrophobic polypeptides (3.2–4.2 kDa) have been identified in the cyt $b_6f$-complex only. These are PetG, PetL, PetM, and PetN, each consisting of little more than a transmembrane helix (Figure 2). In the crystal structure they are found as a transmembrane four-helix bundle, distal to the symmetry axis of the dimer (Figure 3).

## Cytochrome *f*

Mature cyt *f* is a 31 kDa protein built from ~290 amino acids. It holds the heme *c* covalently bound to the pentapeptide CxxCH in an elongated, peripheral domain, largely of $\beta$-sheet structure. This domain is exposed to the intrathylakoid surface, which becomes positively charged during illumination (p-side, s. Figure 1), and is anchored by a C-terminal transmembrane helix. The fifth ligand to the heme iron is an N-atom of a histidine, while the sixth ligand is exceptional for a *c*-type cytochrome: it is the free $\alpha$-amino group of the N-terminal tyrosine (Figure 2).

## Cytochrome $b_6$ + Subunit IV

These two subunits are integral membrane proteins, spanning the membrane seven times together. Cyt $b_6$ (24 kDa, 215 amino acids) contains four hydrophobic helices, the second and fourth holding the two hemes *b* by four H-residues in transmembrane arrangement (Figure 2). These histidines are strictly conserved in all cyt *bc*-complexes. Subunit IV (17 kDa, 160 amino acids) comprises three transmembrane helices. These correspond to helices 5–7 in cyt *b* (42 kDa) from $bc_1$-complexes, which spans the membrane 8 times. The eighth helix is missing in subunit IV.

In addition to the two hemes *b*, cyt $b_6$ contains a third heme, which came as a surprise from the crystal structures (Figure 3). It has escaped spectroscopic detection, because it is a low-spin heme. It is of the *c*′-type, being covalently bound by only one of its vinyl groups to C-37 of cyt $b_6$, which is conserved in all $b_6f$-complexes. This covalent binding is responsible for the old, but misinterpreted notion that cyt $b_6$ stains for heme on denaturing SDS-polyacrylamide electrophoresis, in contrast to other cyt's *b*. Noteworthy, this covalently bound heme has been described for the $b_6f$-type complex of the respiratory, Gram-positive bacterium *Bacillus subtilis* by Yu and LeBrun in 1998 already, without wide recognition, however. It may be involved in ferredoxin–plastoquinone reduction, as an additional entry of electrons into the cyt $b_6f$-complex.

The hydrophobic region of cyt $b_6$ and subunit IV furthermore binds a chlorophyll *a* and a carotenoid (Figure 3).

## The Rieske FeS–Protein

This protein with a 2Fe2S cluster of +300 mV has been discovered by John S. Rieske in mitochondria in 1964. The unusually high redox potential for a FeS–protein results from "softer" ligation of one Fe to the N-atoms

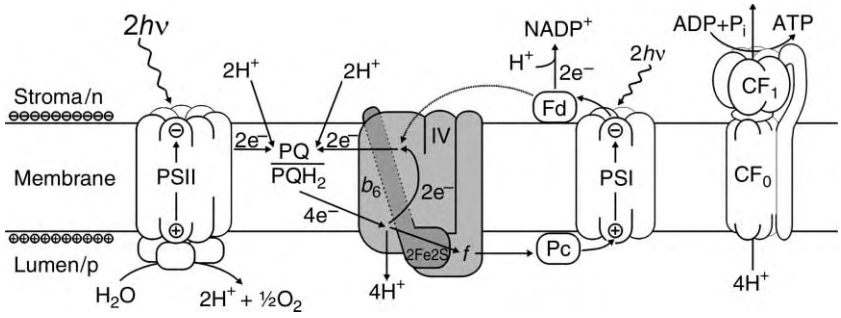



**FIGURE 1** The cytochrome $b_6f$-complex in the chloroplast membrane. The transfer of two electron equivalents from PSII to PSI, and the accompanying proton transfer reactions are shown, indicating the bifurcation of the reduction equivalents from plastoquinol in the Q-cycle of the cyt $b_6f$-complex. Abbreviations: $b_6$, cyt $b_6$; *f*, cyt *f*; 2Fe2S, FeS-cluster of the Rieske protein; IV, subunit IV; PQ, plastoquinone; PC, plastocyanin; Fd, ferredoxin; CF1CFo, $H^+$-translocating ATP synthase; "p" and "n" denote the positively and negatively charged membrane surfaces; the dotted arrow indicates cyclic electron flow around photosytem I.

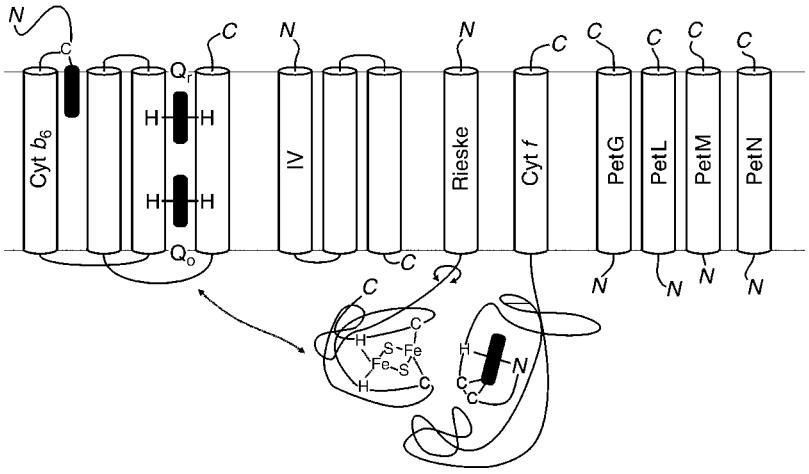



**FIGURE 2** Folding of the protein subunits in the cytochrome $b_6f$-complex across the thylakoid membrane. The solid and dotted double-headed arrows indicate the domain movement of the Rieske FeS–protein by rotation at the "hinge" region. $Q_o$ and $Q_r$ denote the sites of plastoquinol oxidation and plastoquinone reduction, respectively. Italic Ns and Cs stand for N and C termini. Further explanations are given in the text.

of two H-residues, instead of S-atoms of C-residues (Figure 2). The protein of the cyt $b_6f$-complex consists of 179 amino acids (20 kDa). The two cluster binding peptides are CTHLGC and CPCHGSQY, containing a histidine and two cysteins each. The first cystein in each peptide ligates the other Fe-atom, while the second two cysteins form a stabilizing S–S bond. H-bonds from S and Y in the second peptide stabilize the reduced form of the cluster, additionally increasing the redox potential by some 60 mV per H-bond. Rieske proteins of lower redox potentials occur in menaquinol oxidizing organisms, and carry non-H-bonding residues at these positions.

Like the heme $c$ carrying part of cyt $f$, the cluster carrying domain of the Rieske FeS–protein is exposed to the p-surface (Figures 1 and 2), and is tied to the membrane by a single transmembrane helix, at the N terminus. Noteworthy, like in the crystal structures of the cyt $bc_1$-complexes, the Rieske FeS–protein cross-connects the dimer of the cyt $b_6f$-complex, the

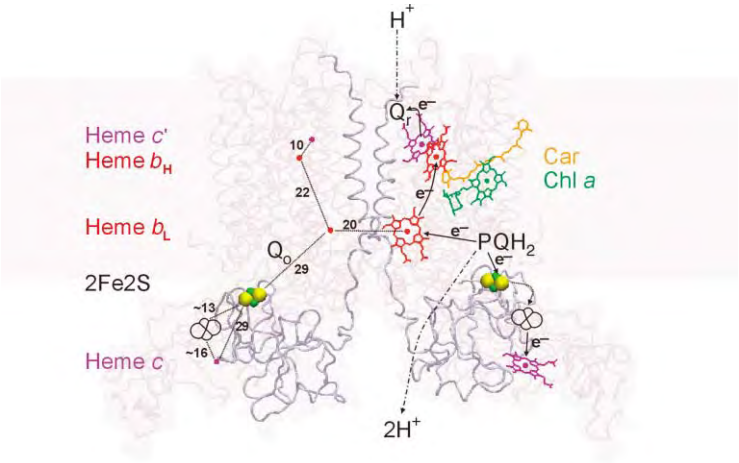



**FIGURE 3** Distances, electron and proton transfer in the crystal structure of the dimeric cytochrome $b_6f$-complex. The figure was constructed from the coordinates for the complex of *Mastigogladus laminosus* submitted to the PDB, accession code 1UM3. The dimeric structure is not totally symmetric, while the one from *Chlamydomonas rheinhardtii* is (PDB accession code 1Q90). Further discrepancies between the two structures are under debate (Daniel Picot, personal communication). In the figure the dimeric structure is depicted in the membrane with its peripheral surface contours and the C$\alpha$-backbone in gray. The backbone of the Rieske FeS–protein which cross connects the dimer is emphasized in darker gray. The 2Fe2S cluster is depicted on both halves, the irons in green and the sulfurs in yellow. Its movement to the position closer to the heme $c$ of cyt $f$ is indicated. The positions of the four hemes are shown with the porphyrin rings on the right, and as the central irons on the left. The distances between the metal centers in numbers of Å are given on the left half, while the right includes the electron and proton transfer steps plus the bound molecules of chlorophyll $a$ (Chl $a$) and carotenoid (Car). Abbreviations: $b_H$ and $b_L$, high and low potential heme $b$ in cyt $b_6$; $Q_o$ and $Q_r$, sites of plastoquinol oxidation and plastoquinone reduction.

FeS-domain being associated with cyt *f* of one-half and the hydrophobic helix with the other (Figure 3). Whether the dimeric structure bears on the function is an unsettled question still. The FeS-domain is linked to the transmembrane helix by a flexible, G-rich "hinge" region, which by rotation allows for the movement of the FeS-domain during the redox cycle. This movement has been documented in oriented samples by polarized EPR, and is indicated by double-headed arrows in Figure 2. The two positions of the FeS-center have been detected in the crystal structure of the mitochondrial cyt $bc_1$-complex by analyzing preparations with and without the specific inhibitor stigmatellin, which locks the conformation into the form with the FeS-center closer to heme $b_L$ of cyt $b_6$, at the Qo-site. Only this position of the FeS-center is seen in the structures of cyt $b_6f$-complex, since the tridecyl derivative of stigmatellin was used to obtain ordered crystals. The second position close to the heme of cyt *f* has been modeled into the cyanobacterial structure, however, and is indicated in Figure 3, which depicts further features of the overall structure. Noteworthy, an electron density close to the heme $c'$ at the Qr-site has been interpreted as bound plastoquinone in the cyanobacterial structure, which has not been identified in the structure for the eukaryotic alga.

## Plastoquinol Oxidation – Q-Cycle and Fes-Domain Movement

In 1960, Britton Chance discovered oxidant-induced reduction of cyt *b* during oxygen pulses of anaerobic mitochondria. A decade later Marten Wikström explained this phenomenon as a "pull–push" effect of ubiquinol oxidation in the cyt $bc_1$-complex, which involves the reactive semiquinone intermediate. Accordingly, Peter Mitchell proposed the Q-cycle mechanism in 1975, which has been reformulated by Antony Crofts, Bernie Trumpower, and others.

The observation of oxidant-induced reduction of cyt $b_6$ in the isolated cyt $b_6f$-complex, with pulses of ferricyanide in the presence of excess plastoquinol, was of key importance for acceptance of an operating Q-cycle also in chloroplasts, because the earlier detection in membrane preparations was complicated by reduction of cyt $b_6$ in cyclic ET around PSI. Quinol oxidation at the $Q_o$-site is the rate-determining step in ET not only because of second order kinetics, but also because it involves the movement of the 2Fe2S-cluster carrying, peripheral domain in the Rieske protein. The Q-cycle mechanism, as indicated in Figures 1 and 3, comprises the following:

1. Two quinone/quinol interaction sites with different semiquinone stability, and accessible from the aqueous space on opposite membrane surfaces, with two pairs of redox centers at two different redox potential levels.
2. At the $Q_o$-site the Rieske FeS-center (+300 mV) oxidizes plastoquinol.
3. The resulting instable semiquinone anion is highly reducing (redox potential of semiquinone anion/plastoquinone ~ − 150 mV), and for kinetic reasons does not deliver its electron to the FeS-center but to nearby low-potential heme *b* (−150 mV) of cyt $b_6$. This bifurcation is the rate-determining step ($\tau \sim$ ms) of the whole cycle, and causes the oxidant-induced reduction of cyt *b*. It is responsible for the transition from 2e- to 1e-transfer in the electron transport chains of photosynthesis and respiration.
4. Furthermore, this step is linked to the movement of the 2Fe2S-cluster towards the heme *c* of cyt *f* (Figures 2 and 3), close enough for efficient electron transfer. At the same time the oxidized plastoquinone is replaced by plastoquinol from the quinol/quinone pool.
5. The electron from low-potential heme *b* moves across the membrane to the high-potential heme *b* (−50 mV), and from there to a plastoquinone molecule at the quinone reduction site ($Q_r$-site), which allows for the electron uptake by stabilizing the resulting semiquinone anion.
6. This semiquinone anion receives a second electron coming from the $Q_o$-site across the membrane via the two heme *b*, takes up two protons from the n-surface and leaves the site as plastoquinol in exchange for plastoquinone.

An alternative view to the Q-cycle is the "semiquinone cycle," in which the instable semiquinone at the $Q_o$-site changes into the stable semiquinone at the $Q_r$-site, either by movement or by conformational dynamics.

In summary, the Q-cycle transports four protons across the membrane per two electrons passing from plastoquinol through the cyt $b_6f$-complex to plastocyanin. Together with the liberation of two protons in water oxidation inside the thylakoids (p-side), and proton uptake by reduction of $CO_2$ via $NADP^+$ outside, a total of six protons are translocated across the chloroplast membrane per 2e if the Q-cycle operates (Figure 1). Evidence for a down-regulation to $4H^+/2e$, i.e., a shut down of the Q-cycle at high-energy pressure across the chloroplast membrane has been provided.

One of the two electrons at the bifurcation point reduces cyt $b_6$, branching off through the membrane. It contributes the "slow phase" to the charge separation across the chloroplast membrane, in addition to the fast phases in the two RCs. These phases are known from the "carotenoid shift," an electrochromic effect on the absorption of carotenoids.

Several inhibitors of electron transport through the cyt $b_6f$-complex are in use, most of them interfering with quinol oxidation at the $Q_o$-site. Among them are the antibiotics stigmatellin and myxothiazol, and the quinone analogues DBMIB (2,5-bromo-3-methyl-6-isopropyl-p-benzoquinone) and UHDBT (5-undecyl-6-hydroxy-4,7-dioxobenzothiazole). As mentioned above, the tridecyl derivative of stigmatellin TDS was used to obtain stable crystals. Antimycin A, a $Q_r$-site inhibitor in cyt $bc_1$-complexes, and which was valuable in elucidating the Q-cycle mechanism, because it stimulates oxidant-induced reduction of cyt $b$ by blocking its reoxidation with quinone, unfortunately is inefficient in the cyt $b_6f$-complex. Possibly the additional heme $c'$ at the $Q_r$-site does not allow the binding of antimycin A. However, MOA-stilbene ($\beta$-methoxacrylate-stilbene) acts on the $Q_r$-site in the cyt $b_6f$-complex, like antimycin A in the cyt $bc_1$-complex. Heptyl- or nonyl-4-hydroxyquinoline N-oxides (HQNO and NQNO) block the quinol/quinone interaction at both sites, with a higher affinity for the $Q_r$-site.

Oxidation of plastoquinol and the coherent movement of the Rieske FeS–protein are rate determining in photosynthetic electron flow, and therefore are linked to regulatory phenomena, like the optimization of the light distribution to the two photosystems via activation of LHCII-kinase (state transitions), but also in light regulation of gene expression.

In the dark and in low light, under aerobic conditions, the chloroplasts are in state 1, with the plastoquinone pool largely oxidized and the outer chlorophyll antenna LHCII associated with PSII for transfer of excitation energy. At high light pressure PSII reduces plastoquinone, and the resulting plastoquinol, via a reduced state of the cyt $b_6f$-complex, activates a specific kinase which phosphorylates LHCII and causes it to move to PS1. Preferential excitation of PS1 (state 2) reoxidizes plastoquinol leading to deactivation of the LHCII-kinase, deposphorylation of LHCII-P by a phosphatase, and the movement of LHCII back to PSII. This way the distribution of the light quanta between the two photosystems is optimally balanced. LHCII-kinase has been found associated with the cyt $b_6f$-complex.

Noteworthy, the electron flow through the cyt $b_6f$-complex *in vivo* can be measured in detail by single turnover laser flash photolysis, or by chlorophyll fluorescence induction (Kautsky-effect) with pulsed light.

## Genes, Biogenesis, and Phylogeny

In cyanobacteria, the genes for the major subunits of the cyt $b_6f$-complex are organized in two transcription units, *petCA* for the Rieske FeS–protein and cyt *f*, and *petBD* for cyt $b_6$ and subunit IV. The genes for the four small subunits PetG, PetL, PetM, and PetN occur in isolated loci. In eukaryotic plants and algae the genes for the Rieske FeS–protein, *petC*, and for one of the small subunits, *petM*, were moved to the nucleus, while the others remained on the chloroplast genome. In vascular plants *petBD* and *petA* were combined with other genes in transcription units, while in green algae like *Chlamydomonas* these genes have been further rearranged and are transcribed independently.

The nuclear genes *petC* and *petM* acquired N-terminal extensions for signal peptides to recognize the machinery for the uptake of the preproteins into chloroplasts. There they are processed by signal peptidases to the mature proteins during insertion into the thylakoid membrane and assembly with the complex. Since the major part of Rieske FeS–protein faces the intrathylakoid surface (p-side), it has to pass the chloroplast envelope as well as the thylakoid membrane on the path from cytoplasmic ribosomes. Thus it is processed in two steps.

Also cyt *f* faces the p-side and has to pass the membrane. Its plastidal gene *petA*, which is transcribed on stromal ribosomes, also carries an N-terminal extension which targets it across the thylakoid membrane where it is processed. The insertion of cyt *f* and of the Rieske FeS–protein into the complex use different pathways. Cyt *f* follows the secA-route known from bacterial secretion, while the assembly of the Rieske FeS–protein is energized by the proton gradient across the membrane (ΔpH-route). The insertion of the hydrophobic proteins cyt $b_6$ and subunit IV into the membrane does not require a signal peptide and may follow yet another route. Interestingly cyt *f* controls the stability and assembly of the Rieske FeS–protein and of the other subunits in the cyt $b_6f$-complex, via a signal in its C terminus which is located on the stromal surface (n-side, see Figure 2). The biosynthesis of 2Fe2S-cluster in the Rieske FeS–protein, and the covalent binding of heme *c* in cyt *f* as well as of the heme $c'$ in cyt $b_6$ are enzymatic processes governed by several nuclear genes. The insertion of the hemes *b* into cyt $b_6$ may occur spontaneously.

In many bacteria the genes of the related cyt $bc_1$-complex, for the Rieske FeS–protein, cyt *b*, and cyt $c_1$ are joined in the tricistronic *fbc*-operon, while in eukaryotes only the gene for cyt *b* remained in mitochondria, the others were transferred to the nucleus where they were furnished with targeting signal peptides.

The Rieske FeS–protein and cyt *b* in the different cyt *bc*-complexes originate from common ancestors, and since they occur in bacteria as well as in archaea, they appear phylogenetically very old. On the other hand cyt *f* and its *c*-type cyt counterparts have various different ancestors. A "cyt $b_6f$-complex" without a cyt *f* has been characterized in Gram-positive bacteria, in

respiratory *Bacilli* and in photosynthetic *Heliobacteria*, a notion which is based on the characteristics of the cyt *b*-complement. These are:

1. the splitting into cyt $b_6$ and subunit IV,
2. 14 instead of 13 amino acid residues between the two histidine ligands of the heme *b* in the fourth transmembrane helix,
3. the loss of the eighth transmembrane helix from subunit IV, and
4. the acquirement of the extra heme $c'$, bound to C-37 of cyt $b_6$.

Interestingly the cyt *b*-complement from the *bc*-complex from green sulfur bacteria fulfills only the second and third of these criteria, but it is not split, and presumably does not contain the extra heme since C-37 is not conserved. Thus the cyt $b_6f$-complex has evolved on the path from the common ancestor of green sulfur bacteria (*Chlorobiaceae*) and Gram-positive bacteria (*Firmicutes*), via cyanobacteria to chloroplasts. The addition of a carotenoid and of a chlorophyll *a* molecule should have occurred after the branching of the cyt $b_6f$-complexes into photosynthetic and respiratory lines.

## See Also the Following Articles

Chlorophylls and Carotenoids • Cytochrome $bc_1$ Complex (Respiratory Chain Complex III) • Cytochrome *c* • Quinones

## Glossary

**archaea** The third domain of life next to eubacteria and eukaryotes, formerly called archaebacteria.

**carotenoid shift** A bathochromic shift of carotenoid absorption in response to an electric field (Stark effect) across the membranes of photosynthetic organisms.

**chlorophyll fluorescence induction** The intensity transient of chlorophyll fluorescence in chloroplasts after onset of illumination (Kautsky effect); the initial rise reflects the reduction of plastoquinone by photosystem II, the difference between maximal and actual fluorescence is related to the reoxidation of plastoquinol by the cyt $b_6f$-complex.

**cyclic electron transport** Electron transport around photosystem I via plastoquinone and the cyt $b_6f$-complex which provides extra ATP.

**LHCII-kinase** An enzyme that phosphorylates LHCII which in that state moves to photosystem I; it is activated by plastoquinol via the reduced state of the cyt $b_6f$-complex.

**light harvesting complex II (LHCII)** Also known as chlorophyll *a/b* binding protein (cab-protein), which constitutes the outer antenna of photosystem II.

**photosystem** The total of pigments assembled around a reaction center.

**Q-cycle** A mechanism of proton translocating quinol oxidation by cyt *bc*-complexes in respiratory and photosynthetic electron transport, first formulated by Peter Mitchell in 1975.

**$Q_o$-site and $Q_r$-site** The sites for quinol oxidation and quinone reduction in cyt *bc*-complexes are synonymous to $Q_p$ and $Q_n$, the sites accessible form the positively and negatively charged membrane surface, respectively. Frequently the pair $Q_o/Q_i$, instead of $Q_o/Q_r$ or $Q_p/Q_n$ is in use, indicating the sites outside and inside the inner mitochondrial and bacterial membrane. Since the sidedness of the thylakoid membrane is turned around the $Q_i$-denotation is not appropriate for the cyt $b_6f$-complex.

**thylakoid** Coined by Wilhelm Menke to denominate the structure of the inner membrane system of the chloroplast which in cross-section appears to be built from stacked "little bags," but their inner space is connected in the third dimension.

**Z-scheme** Electron transport chain of oxygenic photosynthesis pictured in the redox potential scale, first formulated by Robin Hill and Fay Bendall in 1960.

## Further Reading


Allen, J. F. (2002). Plastoquinone redox control of chloroplast protein phosphorylation and distribution of excitation energy between photosystems. *Photosynth. Res.* **73**, 139–148.

Berry, S., and Rumberg, B. (1999). Proton to electron stoichiometries in electron transport of spinach thylakoids. *Biochim. Biophys. Acta* **1410**, 248–261.

Berry, E. A., Guergova-Kuras, M., Huang, L. S., and Crofts, A. R. (2000). Structure and function of cytochrome *bc*-complexes. *Ann. Rev. Biochem.* **69**, 1005–1075.

Brugna, M., Rodgers, S., Schricker, A., Montoya, G., Kazmeier, M., Nitschke, W., and Sinning, I. (2000). A spectroscopic method for observing the domain movement of the Rieske iron–sulfur protein. *Proc. Natl Acad. Sci. USA* **97**, 2069–2074.

Hauska, G. (2003). The isolation of functional cytochrome $b_6f$ complex: From lucky encounter to rewarding experience. *Photosynth. Res.* **120G**, 1–15.

Ke, B. (2001). Photosynthesis – photobiochemistry and photobiophysics. In *Advances in Photosynthesis*, (Govindjee, series ed.) Vol 10/chapter 35, pp. 635–664. Kluwer Academic, Dordrecht, The Netherlands.

Kurisu, G., Zhang, H., Smith, J. L., and Cramer, W. A. (2003). Structure of the cytochrome $b_6f$-complex of oxygenic photosynthesis: Tuning the cavity. *Science* **302**, 1009–1014.

Schütz, M., Brugna, M., Lebrun, E., Baymann, F., Huber, R., Stetter, K. O., Hauska, G., Toci, R., Lemesle-Meunier, D., Tron, P., Schmidt, C., and Nitschke, W. (2000). Early evolution of cytochrome *bc* complexes. *J. Mol. Biol.* **300**, 663–675.

Stroebel, D., Choquet, Y., Popot, J. L., and Picot, D. (2003). An atypical haem in the cytochrome $b_6f$-complex. *Nature* **426**, 413–418.

Whitelegge, J. P., Zhang, H., Aguilera, R., Taylor, R. M., and Cramer, W. A. (2002). Full subunit coverage liquid chromatography electrospray ionization mass spectroscopy (LCMS+) of an oligomeric membrane protein: Cytochrome $b_6f$ complex from spinach and the cyanobacterium *Mastigogladus laminosus*. *Mol. Cell. Proteomics* **1**, 816–827.

Wollmann, F.-A., Minai, L., and Nechushtai, R. (1999). The biogenesis and assembly of photosynthetic proteins in the thylakoid membranes. *Biochim. Biophys. Acta* **1411**, 21–85.

Zito, F., Finazzi, G., Delosme, R., Nitschke, W., Picot, D., and Wollman, F. A. (1999). The Qo site of cytochrome $b_6f$ complex controls the activation of the LHCII kinase. *EMBO J.* **18**, 2961–2969.


## Biography

Günter Hauska is a Professor of Cell Biology and Plant Physiology at the Institute of Botany, in the Department of Biology at the University of Regensburg, Germany. He obtained his Ph.D. in Chemistry at the

University of Vienna in 1967. Subsequently he served as Postdoctoral Fellow in the Biochemistry Department of the Cornell University, before qualifying as Dozent for Biochemistry at the Ruhr-Universität in Bochum, Germany. His research interest is in energy transduction by membrane protein complexes of electron transport chains, especially in quinol oxidation by cytochrome *bc*-complexes and in photosynthetic reaction centers. In recent years he turned to flavoproteins. He is a member of the German Society for Biochemistry and Molecular Biology.

Thomas Schödl graduated in chemistry at the University of Regensburg and finished his Ph.D. in Biochemistry. He is studying the structure and redox reactions of flavoproteins, in particular of the sulfide quinone reductases from bacteria and eukaryotes.

# Cytochrome $bc_1$ Complex (Respiratory Chain Complex III)

**Bernard L. Trumpower**
*Dartmouth Medical School, Hanover, New Hampshire, USA*

**The cytochrome $bc_1$ complex (ubiquinol:cytochrome *c* oxidoreductase complex, E.C. 1.10.2.2) is an energy-transducing, electron transfer enzyme located in the inner mitochondrial membrane of oxygen utilizing eukaryotic cells, where it participates in cell respiration. A functionally similar but structurally simpler version of the $bc_1$ complex is located in the plasma membrane of many, but not all, bacteria, where it takes part in respiration, denitrification, nitrogen fixation, and cyclic photosynthetic electron transfer, depending on the species. In all of these organisms the $bc_1$ complex oxidizes a membrane-localized quinol and reduces a water-soluble, *c*-type cytochrome and links this redox reaction to translocation of protons across the membrane in which the $bc_1$ complex resides. The $bc_1$ complexes from mitochondria of several species have been crystallized and the mechanism of the enzyme is generally well understood, although some questions remain outstanding.**

## Function of the $bc_1$ Complex

The cytochrome $bc_1$ complex participates in respiration in oxygen utilizing cells and also participates in electron transfer in numerous bacteria that utilize alternative terminal-electron acceptors in addition to oxygen. The functional relationship of the $bc_1$ complex to other redox enzymes in these linked electron-transfer systems is illustrated in Figure 1. In mitochondria the $bc_1$ complex is a confluence point for reducing equivalents from the various dehydrogenases and it is essential for mitochondrial respiration, since there is no alternative route to oxidize ubiquinol by molecular oxygen. Bacteria such as *Paracoccus denitrificans* that have a $bc_1$ complex usually have alternative mechanisms to oxidize ubiquinol or otherwise bypass the $bc_1$ complex. Consequently, the enzyme is not essential in these organisms. Hydroxyquinone analogues of ubiquinone, such as atovaquone, inhibit the $bc_1$ complex and are used therapeutically against fungal (e.g., pneumocystis) and parasitic (e.g., malaria) infections, but are ineffective against bacteria in which the enzyme is not essential. In some photosynthetic bacteria, such as *Rhodobacter*, the $bc_1$ complex is essential for cyclic photosynthetic electron transfer in the absence of a terminal electron acceptor, but is not essential when the cells are growing heterotrophically. In all of these organisms the $bc_1$ complex is located in the organelle or plasma membrane and converts the energy associated with electron transfer from ubiquinol to cytochrome *c* into an electrochemical proton gradient across the membrane in which the enzyme resides. The resulting protonmotive force is used by the cell for energy-requiring reactions, including ATP synthesis, ion and metabolite transport, and flagellar motion.

## Structure, Composition, and Properties of the $bc_1$ Complex

### Protein Subunits

All cytochrome $bc_1$ complexes contain three protein subunits with redox prosthetic groups, a diheme cytochrome *b* containing a relatively high-potential $b_H$ heme and a lower potential $b_L$ heme, cytochrome $c_1$, and a Rieske iron–sulfur protein with a 2Fe–2S cluster. In some bacteria, such as *Paracoccus denitrificans* and *Rhodospirillum rubrum*, the $bc_1$ complex contains only these three subunits. Other bacteria, including the *Rhodobacter*, contain a fourth subunit of unknown function that lacks prosthetic groups. *Rhodobacter* can adapt to grow photosynthetically when this subunit is absent, but the stability of the enzyme is compromised, suggesting at least a structural role.

The cytochrome $bc_1$ complexes of mitochondria contain as many as seven or eight supernumerary subunits. The enzyme from *Saccharomyces cerevisiae* contains ten subunits, while those from bovine heart and *Schizosaccharomyces pombe* contain 11. The difference between the 10- and 11-subunit enzymes is due to the fact that the 11-subunit enzymes contain one small subunit that is formed when the presequence that targets the Rieske protein to the mitochondria is cleaved from the protein. The cleaved presequence is retained in the

 © 2004, Elsevier Inc. All Rights Reserved. 

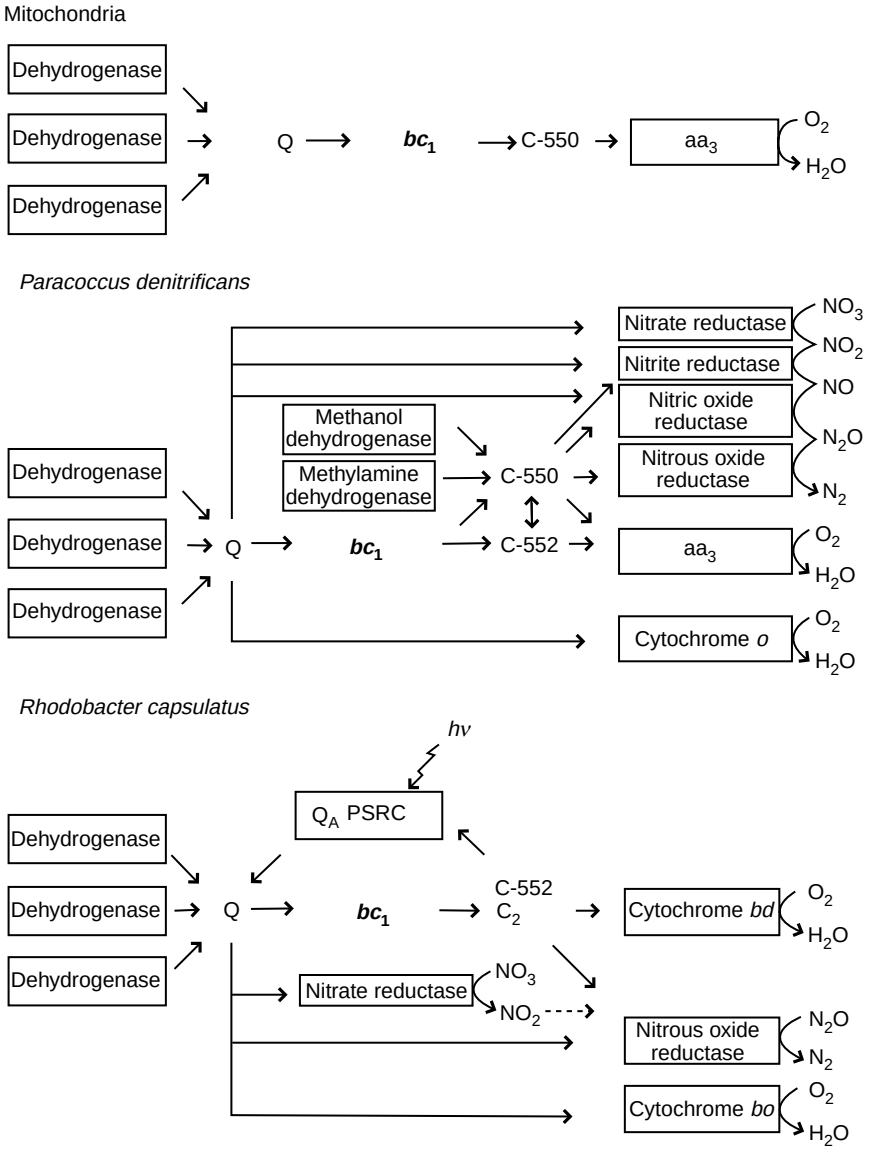



**FIGURE 1** Function of the cytochrome $bc_1$ complex in mitochondria and bacteria. In mitochondria the $bc_1$ complex oxidizes ubiquinol that is formed by reduction of ubiquinone by various dehydrogenases including glycerol phosphate dehydrogenase, succinate dehydrogenase, NADH dehydrogenase, and ETF dehydrogenase. In bacteria such as *Paracoccus dentrificans*, ubiquinol can be oxidized by various routes that bypass the $bc_1$ complex, including a terminal oxidase, cytochrome o, which oxidizes ubiquinol directly, without the intervention of cytochrome *c* and cytochrome *c* oxidase. These bacteria can also use alternative terminal electron acceptors, such as nitrate, in which case the $bc_1$ complex is also not essential for electron transfer from the quinol. In photosynthetic bacteria such as the *Rhodobacter* the $bc_1$ complex participates in cyclic electron transfer when the bacteria are grown photosynthetically, and is essential under those growth conditions. However, *Rhodobacter* can also grow heterotrophically on a variety of nutrients and can use either oxygen or nitrogen in the form of nitrate and nitrous oxide as terminal electron acceptor. Under these conditions the ubiquinol that is formed by the various dehydrogenases can be oxidized by the $bc_1$ complex and electrons transferred via the *c* cytochromes to oxygen or nitrous oxide, or electrons from the quinol can bypass the *c*-cytochrome pool and be transferred directly to nitrate, nitrous oxide, or oxygen.

$bc_1$ complex as an additional subunit. In *Saccharomyces cerevisiae*, the presequence is cleaved from the Rieske protein in two steps and degraded, thus that $bc_1$ complex contains only 10 subunits.

The functions of the supernumerary subunits in the mitochondrial enzymes are not known. They are not required for the electron transfer and proton translocation activities of the enzyme, since the three-subunit enzyme from *Paracoccus* has the same electron transfer and proton translocation functionality as the mitochondrial enzymes. Possible functions for these nonredox subunits include docking sites for ternary complex formation with the dehydrogenase and oxidase complexes, regulation of half-of-the-sites activity of the

dimeric enzyme, structural stability in the relatively long-lived eukaryotes, and protection against oxygen radicals.

## Spectroscopic and Thermodynamic Properties of the $bc_1$ Complex

The $bc_1$ complex is visibly red due to the cytochromes. Optical spectra of the yeast enzyme in the visible region of the spectrum are shown in Figure 2. When the enzyme is reduced by dithionite, a difference spectrum of the dithionite reduced versus ferricyanide oxidized enzyme exhibits an absorption maximum at 562 nm due to ferro-cytochrome $b$ and a shoulder at ~553 nm due to ferro-cytochrome $c_1$. When the enzyme is reduced with ascorbic acid, the reduced versus ferricyanide oxidized difference spectrum consists of only cytochrome $c_1$, with a maximum at 553 nm (Figure 2B). The extinction coefficient of reduced versus oxidized cytochrome $c_1$ at 553 nm versus 539 nm is 17.5 $mM^{-1}\,cm^{-1}$, which was obtained from the purified protein. By subtracting the spectrum of $c_1$ from that of $b+c_1$, it is possible to derive a calculated difference spectrum of the cytochrome $b$ as shown in Figure 2C. The extinction coefficient of reduced versus oxidized cytochrome $b$ at 562 versus 575 nm is 25.6 $mM^{-1}\,cm^{-1}$, which was determined by measuring the fluorescence quenching upon stoichiometric binding of antimycin to the bovine enzyme. This value is an average of the two $b$ hemes. The individual extinction coefficients of the $b_H$ and $b_L$ hemes have not been determined for most $bc_1$ complexes, but the contribution from the $b_H$ heme at 562 is significantly greater than that from the $b_L$ heme, which has a split absorption maximum with peaks at 564–566 and ~558 nm.

The Rieske iron–sulfur protein is a pale yellow-gold color when isolated from the $bc_1$ complex in the oxidized form but bleached to colorless when reduced and thus makes no contribution to the optical spectrum of the reduced enzyme. The redox status of the 2Fe–2S cluster is monitored by electron paramagnetic resonance (EPR) spectroscopy since the reduced cluster is paramagnetic. The Rieske cluster exhibits a unique EPR spectrum with peaks at approximately $g_x = 1.76$, $g_y = 1.90$, and $g_z = 2.03$, resulting in $g_{av} = 1.90–1.91$, which is atypical of binuclear iron–sulfur clusters. The unusual EPR spectrum of the Rieske protein is due to bis-histidine coordination of one of the two iron atoms, which was first suggested by spectroscopic studies and confirmed by crystal structures of the enzyme.

The midpoint potentials of the redox centers in the $bc_1$ complex determine the thermodynamic parameters and in some cases control electron transfer reactions within the enzyme. In *Saccharomyces cerevisiae* the midpoint potentials at pH 7 are as follows: cytochrome $c_1$, +240 mV; Rieske iron–sulfur cluster, +280 mV; cytochrome $b_H$, +120 mV; and cytochrome $b_L$, −30 mV. Although there are species differences in these midpoint potentials it is generally true that the midpoint potential of the Rieske center is ~20–40 mV more positive than that of $c_1$, and that of the $b_L$ heme is 60–120 mV lower than that of the $b_H$ heme. The midpoint potentials of the $b$ hemes are influenced by the detergents used to purify the enzyme, and may be similarly influenced by bound phospholipids. The midpoint potential at pH 7 of ubiquinone is +90 mV in the membrane, but this value may be altered by preferential binding of either quinone or quinol to reaction sites in the enzyme. These midpoint potentials result in a thermodynamic profile of the $bc_1$ complex as shown in Figure 3.

## Three-Dimensional Structure of the $bc_1$ Complex

Cytochrome $bc_1$ complexes from bovine, chicken, and yeast mitochondria have been crystallized and their structures solved to atomic resolution. The yeast enzyme, shown in Figure 4, is the highest resolution structure available at this time. The mitochondrial $bc_1$

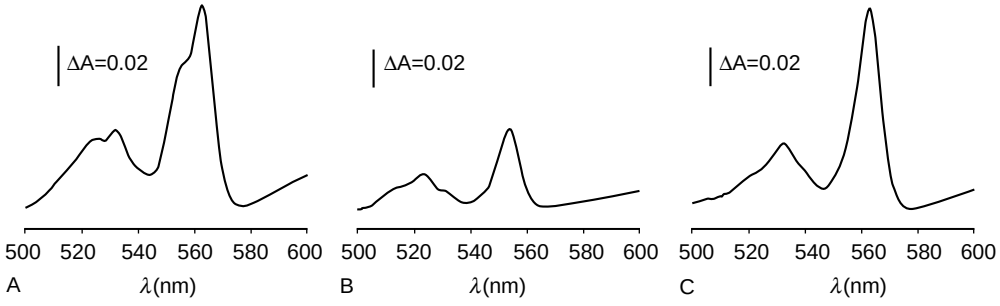



**FIGURE 2** Optical spectra of the yeast cytochrome $bc_1$ complex. Difference spectra of (A) the dithionite reduced versus ferricyanide oxidized enzyme and (B) the ascorbate reduced versus ferricyanide oxidized enzyme. (C) Calculated difference spectrum, obtained by subtracting the spectrum of the ascorbate reduced enzyme from the spectrum of the dithionite reduced enzyme.

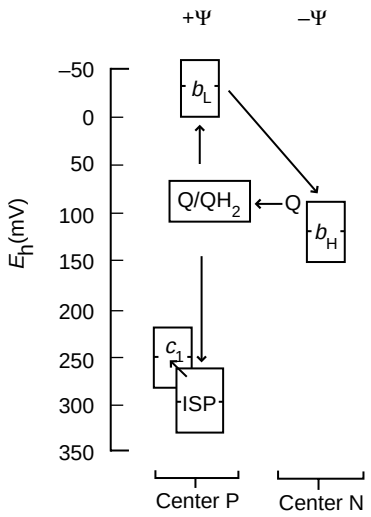



**FIGURE 3** Thermodynamic profile of the *Saccharomyces cerevisiae* cytochrome $bc_1$ complex. The figure illustrates the thermodynamic relationship between the redox components of the cytochrome $bc_1$ complex. Arrows indicate electron transfer reactions in the enzyme. The redox groups are arranged vertically according to their oxidation–reduction potentials, with the center of the boxes positioned vertically at the midpoint potentials of the redox groups. The open boxes depict the approximate range of potentials spanned by the redox components as their oxidation–reduction status varies in response to changes in rates of electron transfer. The midpoint potentials of the redox components are cytochrome $b_L$, −30 mV, cytochrome $b_H$, +120 mV, cytochrome $c_1$, +240 mV, and Rieske iron–sulfur protein, +280 mV.

complex is a symmetrical, oligomeric dimer, in which the three redox subunits are surrounded by a periphery of nonredox, supernumerary subunits. The dimeric structure of the enzyme was suspected from hydrodynamic measurements and confirmed by the first crystal structure of the bovine enzyme. Although the bacterial enzyme has not yet been crystallized, it will presumably turn out to be a dimeric structure too, but lacking the large number of supernumerary subunits.

One unusual aspect of the dimeric structure is that the iron–sulfur protein spans the dimer. The Rieske protein is anchored in one monomer by a single transmembrane helix, as shown in Figure 4A, while the peripheral domain that contains the 2Fe–2S cluster is located in the other monomer, where it forms part of the ubiquinol oxidation site. Crystal structures of the chicken $bc_1$ complex in the absence and presence of stigmatellin, an inhibitory ligand, also provided striking evidence that the peripheral domain of the Rieske protein moves back and forth between positions proximal to cytochrome *b* and cytochrome $c_1$. This suggested that movement of the Rieske protein facilitates electron transfer, which was confirmed by site-directed mutagenesis studies that demonstrated that such movement of the Rieske protein was essential for enzyme activity.

Functionally the enzyme consists of the cytochrome *b* and $c_1$ subunits from one monomer and the Rieske protein from the other, as shown in Figure 4B. The crystal structures established the location of the sites of ubiquinol oxidation and ubiquinone reduction at topographically separated sites within the enzyme. The structures also confirmed the transmembrane disposition of the *b* hemes, which form a conduit through which electrons are cycled across the membrane in which the enzyme resides. These structural details provided the final confirmatory evidence of the protonmotive Q cycle mechanism of the enzyme.

## Mechanism of the Enzyme

### The Protonmotive Q Cycle

The $bc_1$ complex oxidizes ubiquinol and transfers two electrons to two molecules of cytochrome *c*. During this electron transfer reaction two protons are taken up from the mitochondrial matrix or bacterial cytoplasm and four protons are deposited on the other side of the membrane. The mechanism by which the $bc_1$ complex links the electron transfer and proton translocation reactions is the protonmotive Q cycle, shown in Figure 5. In the Q cycle mechanism proton translocation is the net result of topographically segregated reduction of quinone and reoxidation of quinol on opposite sides of the membrane, with protons being carried across the membrane as hydroxyl hydrogen atoms on the quinol. The sites where quinone is reduced and quinol is oxidized are referred to as center N and center P, respectively, since they are located toward the electronegative and electropositive sides of the membrane. Protons are taken up at center N, carried across the membrane by the quinol, and released at center P.

In the first step of the Q cycle, ubiquinol is oxidized at center P in a reaction that divergently transfers the two electrons from ubiquinol to the Rieske iron–sulfur cluster and the cytochrome $b_L$ heme (reactions 1a–1c in Figure 5). In reaction 2 the reduced Rieske cluster oscillates to within electron transfer distance of cytochrome $c_1$ and an electron is transferred from the iron–sulfur cluster to the $c_1$ heme. Two protons are released from center P coincident with ubiquinol oxidation. In reaction 3 an electron is transferred from the $b_L$ to $b_H$ heme, which in turn reduces ubiquinone to ubisemiquinone (reaction 4). Following oxidation of a second ubiquinol at center P and reduction of the *b* cytochromes the $b_H$ heme reduces ubisemiquinone to ubiquinol (reaction 5), accompanied by uptake of two protons at center N.

There is evidence that the mitochondrial $bc_1$ complex functions by an alternating-sites mechanism, in which binding of ubiquinol in one monomer exerts negative cooperativity on binding of ubiquinol in the other

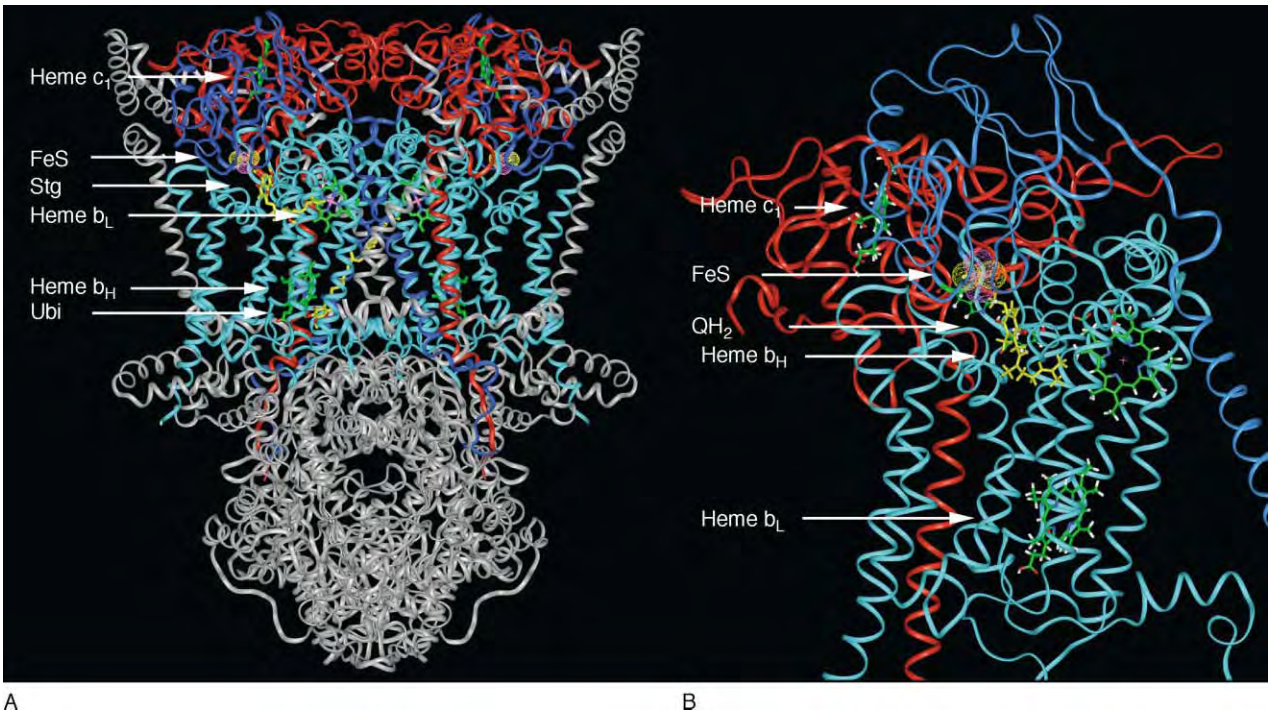



**FIGURE 4** Crystal structure of the cytochrome $bc_1$ complex. The structure in (A) is of the yeast $bc_1$ complex with stigmatellin bound, resolved to 2.3Å by Hunte and co-workers, and shows all of the subunits of the enzyme dimer, except subunit 10, which is lost during purification. The structure is oriented so that the mitochondrial matrix would be at the bottom and the intermembrane space at the top of the figure. Cytochrome *b* is colored cyan, cytochrome $c_1$ red, and the Rieske protein blue. The supernumerary subunits are colored gray. The heme groups (heme $b_L$ and heme $b_H$) are shown as stick structures, colored green. Stigmatellin (Stg) bound at center P in only one monomer is shown as a stick structure, with carbon atoms colored yellow and oxygen atoms red. Ubiquinone (Ubi) bound at center N in only one monomer is also shown as a stick structure, with carbon atoms colored yellow and oxygen atoms red. The iron–sulfur cluster (FeS) is shown with its van der Waals radius, with iron atoms colored purple and sulfur atoms colored yellow. The structure in (B) is of a functional monomer, consisting of cytochrome *b* and cytochrome $c_1$ from one monomer and the Rieske protein from the other. Ubiquinol ($QH_2$) containing two isoprenyl groups is hydrogen bonded between His181 of the Rieske protein and Glu272 of cytochrome *b* to form a putative electron-donor complex. An energy-minimized structure of the docked quinol was obtained by replacing stigmatellin in the crystal structure, followed by molecular dynamics and energy minimization calculations. The protein subunits, heme groups, and ubiquinol are colored as in (A).

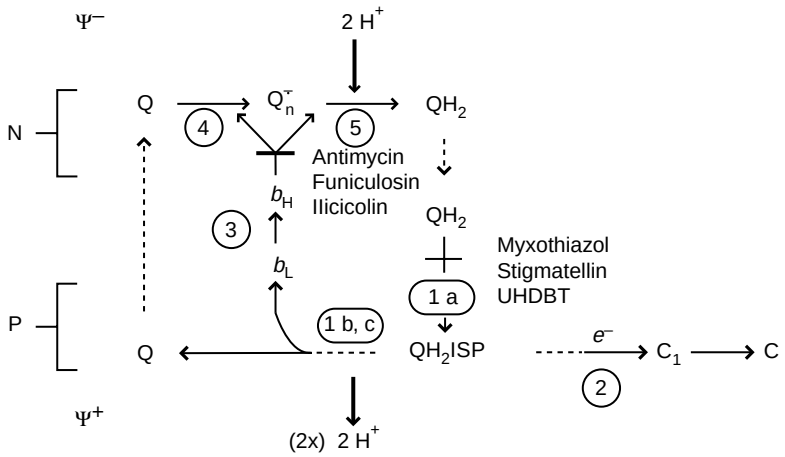
monomer. If the enzyme can be switched from a form in which ubiquinol oxidation alternates between the two monomers to a form where both monomers are simultaneously active, it would provide a mechanism to regulate the activity of the enzyme, perhaps in response to cellular energy needs. Whether such a regulatory mechanism exists and, if so, how it operates, is not known.

## Proton Conduction Pathways

In the Q cycle mechanism, protons are carried across the membrane as hydrogen atoms on the hydroxyl groups of ubiquinol. However, the sites where ubiquinol is oxidized at center P and ubiquinone is reduced at center N are not freely accessible to the bulk aqueous phase at the membrane surface. Consequently, the linkage of proton chemistry to electron transfer requires mechanisms for moving protons to and from the aqueous phase and the hydrophobic sites of quinol and quinone redox reactions.

The crystal structures of the $bc_1$ complex with bound stigmatellin (Figure 4A) show that the inhibitor is hydrogen bonded between His181 (in the yeast numbering system) of the Rieske protein, which is a ligand to the iron-sulfur cluster, and Glu272 of cytochrome *b*. Since stigmatellin presumably mimics an intermediate in ubiquinol oxidation, this suggests that ubiquinol forms an electron-donor complex involving hydrogen bonds between the quinol hydroxyl groups and these two amino acids, as shown in Figure 4B.

When ubiquinol reduces the Rieske protein, an electron is transferred to the iron–sulfur cluster, while a proton from the quinol hydroxyl group protonates the imidazole nitrogen on His181 of the Rieske protein. The Rieske protein acts as a hydrogen carrier and releases the proton to the aqueous phase when it moves proximal to cytochrome $c_1$ and is oxidized. The second proton from ubiquinol is transferred to Glu272 and then to a propionate of the cytochrome *b* heme as an electron is transferred to the $b_L$ heme. From the crystal structure of the yeast enzyme it appears that this proton can then




(1a) $QH_2 + ISP \rightleftharpoons QH_2ISP$

(1b) $QH_2\ ISP + b_L \rightleftharpoons QH_2ISP\ b_L$

(1c) $QH_2\ ISP\ b_L \rightleftharpoons Q + ISP^-H^+ + b_L^-H^+$

(2) $ISP^-H^+ + c_1 \rightleftharpoons ISP + c_1^- + H^+$

(3) $b_L^-H^+ + b_H \rightleftharpoons b_L + b_H^- + H^+$

**FIGURE 5** Mechanism of electron transfer through the cytochrome $bc_1$ complex. The scheme shows the pathway of electron transfer from ubiquinol to cytochrome *c* through the redox centers of the cytochrome $bc_1$ complex. The circled numbers designate electron transfer reactions. Dashed arrows designate movement of ubiquinol ($QH_2$) or ubiquinone (Q) between center P on the positive side of the membrane and center N on the negative side of membrane, and movement of the iron–sulfur protein between cytochrome *b* and cytochrome $c_1$. Solid black bars indicate sites of inhibition by antimycin, funiculosin, ilicicolin, myxothiazol, UHDBT, and stigmatellin. In reaction 1, ubiquinol oxidation delivers two electrons divergently to the Rieske iron–sulfur cluster and the $b_L$ heme, two protons are released, and the resulting ubiquinone leaves center P. In reaction 2, the reduced Rieske cluster oscillates to within electron transfer distance of cytochrome $c_1$, resulting in electron transfer from the iron–sulfur cluster to the $c_1$ heme. In reaction 3, an electron is transferred from the $b_L$ to $b_H$ heme, which in turn reduces ubiquinone to ubisemiquinone (reaction 4). Following oxidation of a second ubiquinol at center P and reduction of the *b* cytochromes the $b_H$ heme reduces ubisemiquinone to ubiquinol (reaction 5), accompanied by uptake of two protons at center N.

access the aqueous surface from the $b_L$ heme via a conserved arginine (Arg79). In this manner two protons are released from center P with minimal charge separation as the two electrons are divergently transferred from the quinol to the high potential and low-potential electron acceptors (Figure 3).

The crystal structure of the yeast enzyme has also revealed pathways for conduction of two protons into center N. One of these pathways, the (CL)/K pathway, involves a conserved lysine and a bound cardiolipin, which may function as a buffer to concentrate protons at the entrance to the pathway. The second proposed proton conduction pathway at center N is referred to as the E/R pathway and involves a conserved glutamate and a conserved arginine. The high resolution of the yeast crystal structure also reveals that one or two bound water molecules may serve as direct proton donors during quinone reduction. Notably, whereas ubiquinol oxidation at center P proceeds with minimal charge separation, ubiquinone reduction at center N begins with substantial charge separation as electrons and protons enter the reaction site from opposite directions, and the redox reaction is accompanied by charge compensation, which enhances the reaction rate.

## Inhibitors

There are numerous inhibitors that act on the cytochrome $bc_1$ complex and that bind specifically to either the ubiquinol oxidation site at center P or the ubiquinone reduction site at center N. These have proven to be especially useful in establishing the mechanism of the enzyme and for selectively blocking electron transfer reactions at either of the two reaction sites in the enzyme to facilitate kinetic analysis. In addition, some of the inhibitors have antifungal and antiparasitic activity.

Antimycin, funiculosin, and ilicicolin H are produced by micro-organisms as defensive toxins and inhibit the $bc_1$ complex by binding to center N with near stoichiometric affinity. Under conditions of catalytic turnover, they block reduction of ubiquinone by cytochrome *b* by

binding to the ubiquinone reduction pocket at a site proximal to the $b_H$ heme, causing electrons to accumulate in the *b* hemes. Under presteady-state conditions where the enzyme is reduced by ubiquinol they block reduction of the $b_H$ heme by quinol.

Stigmatellin, also produced by a micro-organism, inhibits the $bc_1$ complex by binding to the ubiquinol oxidation pocket, simultaneously forming hydrogen bonds to the imidazole nitrogen of His181 on the Rieske protein and a carboxyl oxygen of Glu272 on cytochrome *b*. In this manner the inhibitor locks the Rieske protein in a conformation proximal to cytochrome *b*. Stigmatellin raises the midpoint potential and shifts the EPR spectrum of the iron–sulfur cluster, indicative of its interaction with the Rieske protein.

Hydroxyquinones such as UHDBT, a benzoxythiazole (3-undecyl-2-hydroxy-1,4-benzoxythiazole) and atovaquone, a hydroxynaphthoquinone (2-[trans-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4 hydroxynaphthoquinone) also bind to the ubiquinol oxidation pocket and form a hydrogen bond to His181 of the Rieske protein. Like stigmatellin these ligands raise the midpoint potential and change the EPR spectrum of the iron–sulfur cluster. However, the hydroxyquinone inhibitors do not hydrogen bond to Glu272 of cytochrome *b*. Instead, the crystal structure of the yeast $bc_1$ complex with an analogue of UHDBT bound shows Glu272 rotated toward the heme propionate, which suggested the proton conduction pathway described above.

Methoxyacrylates, such as myxothiazol, also bind to the ubiquinol oxidation pocket but do not interact directly with the Rieske protein. These inhibitors bind proximal to the $b_L$ heme and affect its spectral and thermodynamic properties.

## See Also the Following Articles

Heme Proteins • Heme Synthesis • JAK-STAT Signaling Paradigm • Membrane-Associated Energy Transduction in Bacteria and Archaea • Mitochondrial DNA • Respiratory Chain and ATP Synthase • Ubiquitin-Like Proteins

## Glossary

**protonmotive Q cycle** The mechanism of electron transfer by which the cytochrome $bc_1$ complex transfers electrons from ubiquinol to cytochrome *c* and links the electron transfer to proton translocation. The mechanism is so-named, because the enzyme oxidizes ubiquinol on one side of the membrane and re-reduces ubiquinone on the other side in a cyclic manner, thus bringing about transmembrane movement of protons, carried through the membrane as hydrogen's on the quinol hydroxyl groups.

**ubiquinone** 2,3-dimethoxyl-5-multiprenyl-6-methyl-1,4-benzoquinone, where the multiprenyl group is an isoprenoid side chain consisting of 6–10 isoprenyl groups, is a lipid-soluble benzoquinone that is reduced to quinol by various dehydrogenases and reoxidized to quinone by the cytochrome $bc_1$ complex.

## Further Reading

Hunte, C., Koepke, J., Lange, C., Roßmanith, T., and Michel, H. (2000). Structure at 2.3 angstrom resolution of the cytochrome $bc_1$ complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment. *Structure* **8**, 669–684.

Link, T. A. (1999). The structures of Rieske and Rieske-type proteins. *Adv. Inorg. Chem.* **47**, 83–157.

Trumpower, B. L. (1990). The protonmotive Q cycle: Coupling of proton translocation to electron transfer by the cytochrome $bc_1$ complex. *J. Biol. Chem.* **265**, 11409–11412.

Von Jagow, G., and Link, T. A. (1986). Use of specific inhibitors of the mitochondrial $bc_1$ complex. *Methods Enzymol.* **126**, 253–271.

Zhang, Z. L., Huang, L. S., Shulmeister, V. M., Chi, Y. I., Kim, K. K., Hung, L. W., Crofts, A. R., Berry, E. A., and Kim, S. H. (1998). Electron transfer by domain movement in cytochrome $bc_1$. *Nature* **392**, 677–684.

## Biography

Bernard L. Trumpower is a Professor of Biochemistry at Dartmouth Medical School. His principal research interest is the function of the cytochrome $bc_1$ complex. Dr. Trumpower received his Ph.D. from St. Louis University and did his postdoctoral training at Cornell University with Prof. Efraim Racker. Dr. Trumpower has been an established investigator of the American Heart Association and received a Merit Award from the National Institutes of Health. He has authored more than 100 original research articles on the cytochrome $bc_1$ complex, and in 1984 he was awarded the Humboldt Prize by the Federal Republic of Germany for his contributions to understanding the enzymology of respiratory enzymes.

# Cytochrome *c*

**Hans Tuppy and Günther Kreil**
*University of Vienna, Vienna, Austria*

**Cytochrome *c* is a heme protein that is present in and can easily be isolated from mitochondria of all eukaryotic organisms. The amino acid sequence of the protein moiety was among the first sequences which could be elucidated. This was the starting point for comparative studies about sequence variations found in cytochrome *c* from a wide range of species. A phylogenetic tree constructed on the basis of this information was found to be biologically significant and became exemplary for subsequent studies on molecular evolution. The function of cytochrome *c* in the respiratory chain as an electron carrier is well established. More recently, an additional role of cytochrome *c* was discovered: its release from mitochondria into the cytosol triggers apoptosis – the programmed cell death.**

## Introduction

Cytochromes are proteins which contain heme as their prosthetic group and whose principal biological function, in the cells of animals, plants, and microorganisms, is electron transport. The foundations of the knowledge of heme proteins and their roles as electron carriers in cell respiration were laid by David Keilin (1887–1963), ~80 years ago. In the cytochromes, the iron which is coordinately linked with four nitrogens within the prosthetic group and with two additional ligands provided by the protein moiety, can alternate between a reduced $Fe^{2+}$ and an oxidized $Fe^{3+}$ state. Different classes of cytochromes (*a*-type, *b*-type, *c*-type) differ in the nature of their heme prosthetic groups. They can be observed and differentiated spectroscopically, on the basis of characteristic absorption bands. The absorption peaks of reduced cytochrome *c* and other *c*-type cytochromes (such as $c_1$) are at ~550, 520, 416, and 270 nm. The *b*-type and *a*-type cytochromes absorb visible light at higher wavelengths. In eukaryotic cells, cytochromes of types *a*, *b*, and *c* are found predominantly within mitochondria. In the mitochondrial respiratory chain, cytochrome *c* accepts electrons from complex III, which contains cytochromes *b* and $c_1$ ($bc_1$), and transmits them to complex IV (cytochrome oxidase), which has two heme prosthetic groups ($aa_3$). These respiratory complexes, with their respective cytochromes, are firmly integrated in the inner membrane of the mitochondrion. Cytochrome *c*, by contrast, is only loosely bound in the space between the inner and outer mitochondrial membranes and shuttles between $bc_1$ and $aa_3$. As shown by Keilin and others, cytochrome *c* can be easily removed experimentally from and reincorporated into isolated mitochondria, their respiratory capacity thereby being impaired and restored, respectively. It has recently been discovered that the release of cytochrome *c* from mitochondria into the cytoplasm is an important step in programmed cell death. This participation in apoptosis of eukaryotic cells is now considered to be another significant function of cytochrome *c*.

Cytochrome *c* and *c*-type cytochromes occur in all eukaryotic organisms. The comparative analysis of the protein structures of cytochromes isolated from a multitude of different species of organisms has made it possible to establish evolutionary relationships between them. The phylogenetic tree based on the slow changes of the structure of cytochrome *c* in the course of evolution is most impressive and biologically relevant.

The *c*-type cytochromes are also present in prokaryotes, where they may function in a respiratory chain or in photosynthesis. Examples are cytochrome $c_2$ from *Rhodospirillum rubrum* (purple bacteria) and cytochrome $c_{551}$ from *Pseudomonas aeroginosa* (gram-negative bacteria). These are distantly related to mitochondrial cytochromes *c*; however, a more detailed discussion of these diverse hemoproteins is outside the scope of this article.

## Attachment of the Heme Prosthetic Group to Cytochrome *c* Apoprotein

The prosthetic group of *c*-type cytochromes, such as cytochrome *c* and $c_1$ in mitochondria and cytochrome *f* in chloroplasts, unlike that of *a*- and of *b*-type cytochromes, is covalently linked to the polypeptide moiety. The thiol groups of two cysteinyl residues in a Cys-X-Y-Cys-His peptide motif are attached to two vinyl groups of heme through thioether bonds.

 © 2004, Elsevier Inc. All Rights Reserved. 

This linkage is resistant to heat and hydrolysis, but can be broken with the help of silver or mercury salts. Thus, the polypeptide moiety of cytochrome *c*, or of heme peptides derived from it by proteolytic cleavage, could be obtained. In eukaryotic cells, the apoprotein of cytochrome *c* is encoded by a nuclear gene, translated on cytoplasmic ribosomes and translocated into the intermembrane space of mitochondria, where an enzyme, cytochrome *c* heme lyase, combines it with heme. An *in vitro* synthesis of a microbial *c*-type cytochrome from its apoprotein and reduced heme has recently been achieved.

## Amino Acid Sequence Studies

Cytochrome *c* is a small, water-soluble protein that can easily be isolated and purified from cells and tissues of many different eukaryotic organisms. In proteolytic digests of cytochrome *c*, one fragment containing the covalently linked heme group can be separated from all the other ones. Studies on the amino acid sequence of these heme peptides isolated from cytochromes *c* of diverse origin thus became feasible. It turned out that certain sequence elements, like the one mentioned in the introduction were highly conserved from yeast to mammalian cytochromes *c*.

Expanding on this early work, cytochrome *c* from horse heart was one of the first proteins whose complete amino acid sequence could be determined. It consists of a single polypeptide chain of only 104 residues that could be analyzed even with, by modern standards, rather primitive tools available around 1960. The protein contains many (up to 19) lysine residues, whose positive charges can form ionic bonds with constituents of the inner mitochondrial membrane.

With the horse sequence as a reference, cytochrome *c* isolated from diverse species was subsequently analyzed in quick succession. These included other mammals (human, rhesus monkey, pig, dog, rabbit, whale, and kangoroo), birds (chicken, pigeon, king penguin, turtle), reptiles (rattlesnake, alligator), amphibia (bullfrog), fish (tuna, carp, dogfish) and the chordate pacific lamprey. And with unabated pace, additional sequences were determined for cytochromes *c* from flies, moths, yeasts, Neurospora, and a large variety of plants. By now, we know the sequence of this protein from more than 100 eukaryotic species. As a result of these efforts, cytochrome *c* became the first and still is a standard example for studies on the molecular evolution of proteins. Nuclear genes encoding cytochrome *c* have been analyzed from various species. The mammalian gene contains a small intron in the coding sequence and a larger one upstream of the initiation codon.

The recent progress in the sequencing of whole eukaryotic genomes has also yielded information about the number of cytochrome *c* genes in various species. For example, in the human genome, a single gene for cytochrome *c* is present on chromosome 7, along with 49 pseudogenes located on many different chromosomes. These are mostly processed pseudogenes, which were formed by retro-transcription of an mRNA. In rodents, two cytochrome *c* genes are expressed, one of them exclusively in the testis. This latter gene is still present as a pseudogene in the human genome.

## Evolutionary Aspects

Cytochrome *c* is a highly conserved protein, which retained many structural characteristics over the eons of evolution of animals and plants. This makes it an ideal tool for amino acid sequence comparisons over a wide range of species (Figure 1). From the sequence differences, a molecular "pedigree" can be constructed based on the original assumption, now corroborated by many examples, that the longer ago the common

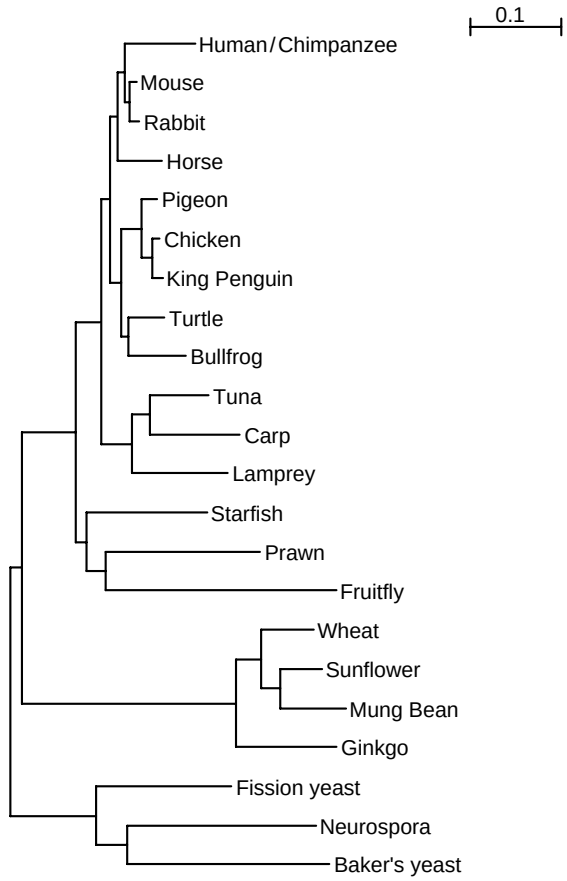



**FIGURE 1** Phylogenetic tree constructed from the amino acid sequences of cytochrome *c* using the ClustalW program. The length of the branches corresponds to evolutionary distances (0.1 = 10% amino acid substitutions).

ancestor of two species existed, the more amino acid changes will have accumulated since. As it turned out, the phylogenetic trees constructed on the basis of the sequence of a single protein were surprisingly similar to those derived from comparative anatomy, fossils etc. Moreover, since cytochrome *c* could be extracted from species for which no reliable data were available from other sources, these could now also be included in this analysis.

In this comparison of cytochromes *c* from diverse eukaryotic species, it was found that more than a quarter of the amino acids have been conserved during more than a billion years of evolution. Among these immutable residues are the two cysteines to which the heme is bound via thioether linkages. The central iron of the planar heme is not only bound to four nitrogen atoms of the porphyrin but, in addition, to the side chains of two invariant amino acids of the protein, one being a histidine adjacent to the second cysteine (residue #18 in the vertebrate sequences), the other the sulfur atom of methionine 80, present on opposite sides. Moreover, certain amino acids with hydrophobic side chains, some of which line the crevice where the heme is buried, are also conserved. Surprisingly, it was found that several glycine residues in the sequence were also invariant. Glycine is the smallest amino acid containing only a hydrogen atom where the 19 others have more or less bulky side chains. From the three-dimensional structure, this first puzzling observation could be explained. At these sites, there is simply no space in this tightly folded protein and every other amino acid would presumably prevent proper folding of the polypeptide chain and thus be deleterious. Also, some charged amino acids, mostly lysines, have been conserved. These may be essential for the ionic interaction with two its reaction partners as well as with acidic phospholipids present in the inner mitochondrial membrane.

## Three-Dimensional Structure

Using X-ray crystallographic analysis, the three-dimensional structure of tuna cytochrome *c* in its oxidized and reduced forms could be determined. As shown by these studies, the polypeptide chain is tightly wrapped around the heme, which sits in a deep pocket, exposed only on one edge to the solvent. In accordance with the "oil droplet" model of folded proteins, the hydrophobic amino acids are mostly buried inside the protein, some of them surrounding the heme. In the respiratory chain located in the inner membrane of mitochondria, cytochrome *c* shuttles between a reduced ferro-($Fe^{2+}$) and ferri-($Fe^{3+}$) form. In this redox cycle, the protein changes its conformation, with the reduced form having a more compact structure.

## Interactions of Cytochrome *c* in the Respiratory Chain

Cytochrome *c* is loosely bound to the outer surface of the inner mitochondrial membrane. In the respiratory chain, an electron is transferred to cytochrome *c* from cytochrome $bc_1$. These cytochromes are part of a large, membrane-bound complex, also termed complex III composed of 11 proteins. Subsequently, the electron is delivered to the cytochrome $aa_3$ of the complex IV, the cytochrome oxidase, which contains 13 proteins. Some of the molecular details of this electron transport has recently also been clarified. In the crystal structure of the yeast cytochrome $bc_1$ complex with its bound substrate cytochrome *c*, the heme groups of cytochrome $c_1$ and *c* are in close proximity (see Figure 2). This suggests that the redox process takes place by direct heme-to-heme electron transfer. The same may also be true for the oxidation of cytochrome *c* by cytochrome $aa_3$.

In both redox reactions, a proton is pumped across the inner membrane. The proton gradient thus formed drives the ATP synthetase motor, which results in the synthesis of ATP.

## Cytochrome *c* and Apoptosis

Cells that are damaged or are no longer needed may undergo a form of cell death that is controlled and executed by intracellular programs. One major apoptotic program involves the release of cytochrome *c* from the intermembrane space of mitochondria into the cytosol, together with some other mitochondrial proteins. Once in the cytoplasm, cytochrome *c* combines with an "apoptosis activating factor-1" (Apaf-1) and thus triggers the assembly of a multimeric protein complex, the so-called apoptosome. An inactive proform of a proteolytic enzyme, procaspase-9, is then recruited to the apoptosome and activated. Active caspase-9, in turn, activates other caspases, whose proteolytic actions finally lead to cell death. The crucial role of cytochrome *c* in this process is shown by the fact that microinjection of cytochrome *c* into the cytoplasm suffices to induce apoptosis in several cell lines. In the interaction with Apaf-1, as in the interaction with cytochrome $c_1$, the exposed heme edge of cytochrome *c* is involved.

When cytochrome *c* molecules are released from the mitochondrion, they have to pass through pores in the outer membrane of the organelle. A group of related proteins, the Bcl-2 family, regulates apoptosis by controlling the permeability of this membrane. Bcl-2 itself and other antiapoptotic proteins are located in the outer mitochondrial membrane and inhibit cytochrome *c* release. Conversely, release of cytochrome *c* is

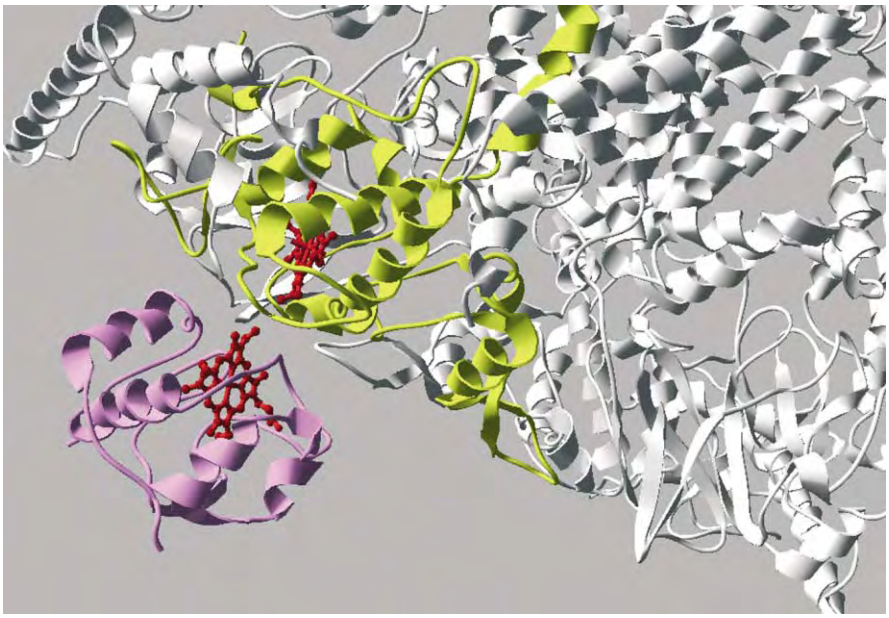


**FIGURE 2** Part of the yeast cytochrome $bc_1$ complex and the bound substrate cytochrome *c* (Protein Data Bank, accession number 1KYO). Cytochrome $c_1$, yellow; cytochrome *c*, magenta; hemes, red.

triggered by proapoptotic members of the Bcl-2 family, which are induced by a number of stimuli to translocate to the mitochondria. Since one of the functions of apoptosis is to help the multicellular organism get rid of cancer cells, the pro- and antiapoptotic Bcl-2 family members are considered to be oncogenic and antioncogenic proteins, respectively. They control whether cytochrome *c*, besides being a vital mediator of electron transfer in respiration, also gets involved in cell death.

## See Also the Following Articles

Cell Death by Apoptosis and Necrosis • Cytochrome Oxidases, Bacterial • Heme Proteins • Heme Synthesis • Purple Bacteria: Electron Acceptors and Donors • Purple Bacteria: Photosynthetic Reaction Centers

## Glossary

**apoptosis** Also called "programmed cell death," a means for the multicellular organism to eliminate cells that are no longer needed or are damaged.

**cytochromes** Literally "cell pigments," heme proteins chiefly involved in cell respiration and energy supply.

**heme** An iron porphyrin complex that is attached (as a "prosthetic group") to protein moieties in the red blood pigment hemoglobin, in the cytochromes and in many other heme proteins.

**mitochondria** Intracellular organelles, the main site of oxidative metabolism which supplies the cell with utilizable energy in the form of ATP. In the course of cellular respiration, elementary oxygen is taken up by mitochondria and reduced to water. The electrons required for reduction are transmitted to oxygen via the respiratory chain, one of the electron transporters being cytochrome *c*.

## Further Reading

Dickerson, R. E. (1972). The structure and history of an ancient protein. *Sci. Am.* (April issue), 58–70.

Dickerson, R. E. (1980). Cytochrome c and the evolution of energy metabolism. *Sci. Am.* (March issue), 98–109.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature* **407**, 770–776.

Lange, C., and Hunte, C. (2002). Crystal structure of the yeast cytochrome $bc_1$ complex with its bound substrate cytochrome *c*. *Proc. Natl. Acad. Sci. USA* **99**, 2800–2805.

Saraste, M. (1999). Oxidative phosphorylation at the *fin de siècle*. *Science* **283**, 1488–1493.

Slater, E. C. (2003). Keilin, cytochromes, and the respiratory chain. *J. Biol. Chem.* **278**, 16455–16461.

Zhang, Z., and Gerstein, M. (2003). The human genome has 49 cytochrome *c* pseudogenes, including a relic of a primordial gene that still functions in mice. *Gene* **312**, 61–72.

## Biography

Hans Tuppy is Professor Emeritus and former Chairman of the Institute of Biochemistry at the University of Vienna, Austria. He worked, among other research topics, on the primary structure and species specificity of cytochrome *c*.

Günther Kreil worked in the Institute of Molecular Biology of the Austrian Academy of Sciences in Salzburg. His early work dealt with the amino acid sequence of horse and tuna cytochrome *c*.

# Cytochrome Oxidases, Bacterial

**Peter Brzezinski and Pia Ädelroth**
*Stockholm University, Stockholm, Sweden*

**Bacterial cytochrome *c* oxidases are integral membrane proteins that catalyze the four-electron reduction of dioxygen ($O_2$) to water and oxidation of different types of water-soluble or membrane-anchored cytochromes *c* (cyt.*c*). The oxygen-reducing site of the enzymes consists of a heme-copper center, which is buried within the protein. Hence, cytochrome *c* oxidases belong to the super-family of heme-copper oxidases, a class of enzymes that also includes the quinol oxidases, which use different types of lipid-soluble quinols ($QH_2$) instead of cytochrome *c* as their electron donor. The heme-copper oxidases are also often termed *respiratory oxidases* or *terminal oxidases*, terms that refer to the enzymes being the last components of the respiratory chains of aerobic organisms. However, not all terminal oxidases belong to the heme–copper oxidase superfamily. One example is the group of so-called alternative oxidases (e.g., in plants), which do not contain any heme groups.**

## Chemical Process

In the cytochrome *c* oxidases the protons used for $O_2$ reduction to water (substrate protons) are taken up from the inner (cytosol) side of the membrane, while cytochrome *c* reacts on the opposite, outer (periplasm) side of the membrane. In the quinol oxidases, the quinol binds from the core of the membrane, and upon oxidation, the protons are released to the periplasm. Thus, the reaction catalyzed by the heme-copper oxidases has a direction not only in time (c.f. any chemical reaction), but also in space, which is referred to as *vectorial chemistry*. The charge distribution across the membrane is such that there are more positive charges on the outer side than on the inner side. Hence, the outer and inner sides are referred to as the positive (*P*) and negative (*N*) sides, respectively:

cytochrome *c* oxidases

$$4\text{cyt.}c_P^{2+} + O_2 + 4H_N^+ \rightarrow 4\text{cyt.}c_P^{3+} + 2H_2O$$

quinol oxidases

$$2QH_2 + O_2 + 4H_N^+ \rightarrow 2Q + 2H_2O + 4H_P^+,$$

where the subscripts *N* and *P* refer to the negative and positive sides, respectively. In both cases outlined above, a net of four positive charges are generated on the *P*-side, while four positive charges are consumed on the *N*-side. Consequently, the chemical reactions catalyzed by heme-copper oxidases are arranged topographically in such a way that they result in a charge separation corresponding to the net transfer of one positive charge from the *N*-side to the *P*-side of the membrane per electron transferred to $O_2$. In addition, for most heme-copper oxidases characterized to date, part of the free energy released in the catalytic reaction is also used to pump (translocate) protons from the *N*-side to the *P*-side of the membrane, with an average stoichiometry of one proton per electron (see Figure 1 and more detailed discussion below). Hence, on average, two charges are transferred across the inner membrane per electron transferred to oxygen. As suggested by Peter Mitchell and formulated in the Chemiosmotic theory, this transmembrane proton and voltage gradient generated in part by the heme-copper oxidases is used, for example, for synthesis of ATP by the ATP synthase.

## Structures

The bacterial heme–copper oxidases consist of several (in many cases four) subunits, located in the (inner) cell membrane of the bacterium. The minimal functional unit of the heme-copper oxidases is the subunit (SU) I–II complex (see also below). The heme–copper oxidase family is defined by the presence in SU I of six conserved histidine residues that coordinate three redox-active metal sites: (1) a six-coordinated heme group which has two axial His ligands and in which the iron ion is in a low-spin state; (2) a five-coordinated heme group with one axial His ligand and in which the iron ion generally is found in the high-spin state; and (3) a copper ion ($Cu_B$) which is bound by three His ligands. The SU I scaffold, which holds the redox centers, comprises at least 12 transmembrane helices, which span the membrane. The five-coordinated heme is usually denoted with a subscript 3, e.g., heme $a_3$. In the examples and discussion

 © 2004, Elsevier Inc. All Rights Reserved. 

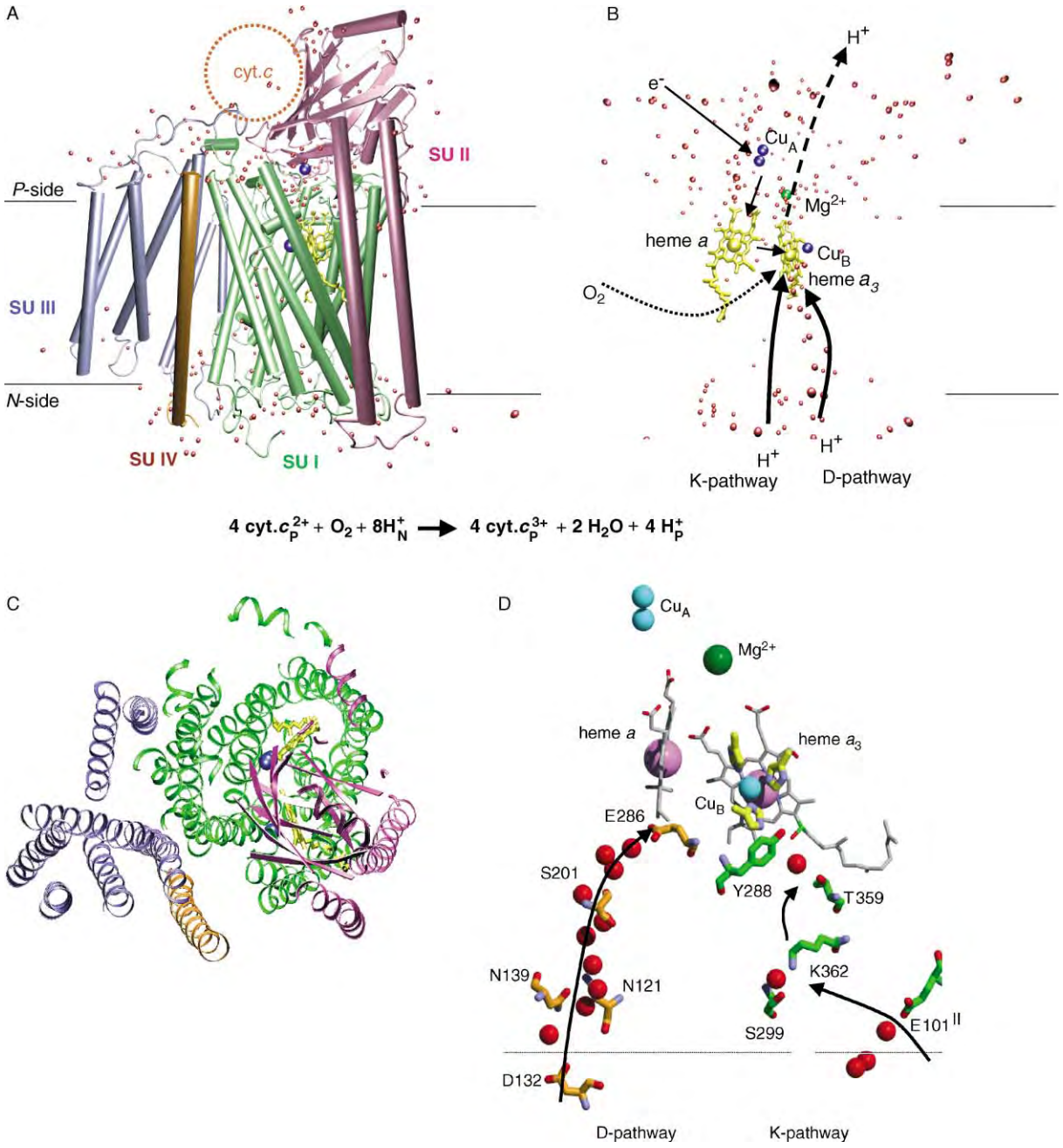



**FIGURE 1** (A) The overall structure of the *R. sphaeroides* cytochrome $aa_3$ (cytochrome *c* oxidase). Subunits I, II, III, and IV are shown in different colors as indicated. The heme groups are shown in yellow, the copper ions in blue, and a redox inactive $Mg^{2+}$ ion in green. Three redox active sites, heme *a*, and the catalytic site consisting of heme $a_3$ and $Cu_B$ are found in SU I. The $Cu_A$ center is found in SU II. A likely location of the cytochrome *c* binding site is indicated. Water molecules resolved in the structure are shown as red spheres. (B) Only the cofactors are shown together with the water molecules resolved in the structure. Substrate and pumped protons (thick arrows) are taken up from the inner, cytosol side (*N*-side) and the pumped protons (dashed arrow) are released on the outer, periplasm side (*P*-side), as shown. $e^-$ is the electron from cyto *c* (thin arrows). (C) Top view of the enzyme. (D) The redox-active cofactors (and the $Mg^{2+}$ ion) and proton-transfer pathways of cytochrome *c* oxidase. The overall reaction catalyzed by cytochrome c oxidase is described by the reaction formula, where the subscripts *P* and *N* refer to the two sides of the membrane. Subunits I–III of the *R. sphaeroides*, *P. denitrificans*, and the mitochondrial enzymes are highly homologous and their overall structures are very similar.

below, we call the two hemes of the enzyme hemes *a* and $a_3$, respectively.

Unlike the six-coordinated heme, heme $a_3$ binds ligands such as $O_2$, CO, and $CN^-$. Heme $a_3$ and $Cu_B$ are collectively called the binuclear center, which is the catalytic site at which $O_2$ is reduced to water. The heme groups may be of different types, such as heme *a*, *b*, or *o*, where the two hemes in SU I may be of the same or of different types. Heme–copper oxidases are also often given a name that indicates the types of hemes present, e.g., cytochrome $aa_3$. Many bacteria have several types of oxidases, which are expressed to various degrees

depending on the environmental conditions (e.g., the $O_2$ concentration). The X-ray structures of heme–copper oxidases that have been determined to date are summarized in Table I.

A common feature of most (but not all) cytochrome *c* oxidases is a copper center ($Cu_A$) bound in SU II, which is the primary electron acceptor from cytochrome *c*. Hence, the binding site for the water-soluble cytochrome *c* is located near $Cu_A$ (see Figure 1). The $Cu_A$ center is composed of two copper ions coordinated by two –SH groups of two Cys residues (among other ligands), which resembles the [2Fe–2S] centers of iron–sulfur proteins. The $Cu_A$ center can accept and donate one electron switching between the formal charges $[Cu^{1+}–Cu^{1+}]$ and $[Cu^{1.5+}–Cu^{1.5+}]$ in the reduced and oxidized form, respectively. Many cytochrome *c* oxidases that do not have the $Cu_A$ site instead have a cytochrome *c* subunit that protrudes into the inter-membrane space and acts as the primary electron acceptor. In the ubiquinol oxidase from *E. coli*, the ubiquinol binding site was suggested from an analysis of the three-dimensional structure and from experiments using site-directed mutagenesis to be found in the membrane-spanning part of SU I at a cluster of polar residues exposed to the interior of the lipid bilayer.

Subunit III does not contain any redox-active cofactors. Even though the enzyme with SU III removed displays $O_2$-reduction activity and pumps protons (although with a stoichiometry of less than one proton per electron), this form of the enzyme becomes inactivated after a limited number of reaction cycles. Consequently, SU III is important for the stability of the protein.

### Proton-Transfer Pathways

As seen in Figure 1, the $O_2$-reducing site is located in the membrane-spanning part of the enzyme. Therefore, proton-transfer pathways, leading from the protein surface on the *N*-side to the binuclear center, are needed. Such pathways are typically composed of polar and protonatable amino-acid residues as well as of water molecules forming a hydrogen-bonded chain. The detailed composition of these pathways varies in the bacterial heme–copper oxidases. Two pathways have been identified from an analysis of the three-dimensional structures of heme–copper oxidases determined to date, from a comparison of the amino-acid residue sequences of a large number of other heme–copper oxidases, and from the combined use of site-directed mutagenesis and functional studies. These pathways are found in SU I and are named the D- and K-pathways, after residues Asp(D)132 and Lys(K)-362, respectively. We use the *R. sphaeroides* cytochrome $aa_3$ amino-acid residue numbering because the structure shown in Figure 1 is from that enzyme. Both pathways start at the inner-side surface and lead to the catalytic site (Figures 1B and 1D). A common feature of a large number of oxidases is a Glu residue in the D-pathway, E(I-286) in the *R. sphaeroides* cytochrome $aa_3$, located about 25Å from D(I-132) and about 10Å from the binuclear center. In heme–copper oxidases in which the Glu is not conserved, other protonatable residues are found at about the same location in space.

## Oxygen Binding and Reduction

Even though the reduction of $O_2$ to water is a highly exergonic reaction, the $O_2$ molecule is kinetically stabilized against reduction due to two factors. First, the one-electron reduction of $O_2$ to form the superoxide radical $O_2^{\cdot-}$ is associated with a positive free energy change, which imposes a thermodynamic barrier in the initial step of the reduction process. Second, in the ground electronic state, $O_2$ has two unpaired electrons (triplet state), which imposes spin restrictions on many of its reactions. The heme copper oxidases overcome

**TABLE I**

**Structures of Heme–Copper Oxidases from the Protein Data Bank (PDB)* (the Bovine Heart Enzyme is also Included)**

| Heme–copper oxidase | Source | PDB code | Resolution (Å) | Comment |
|---|---|---|---|---|
| cytochrome $aa_3$ | *P. denitrificans* | 1AR1 | 2.7 | two-subunit enzyme |
| | | 1QLE | 3.0 | four-subunit enzyme |
| cytochrome $bo_3$ | *E. coli* | 1FFT | 3.5 | |
| cytochrome $ba_3$ | *T. thermophilus* | 1EHK | 2.4 | |
| cytochrome $aa_3$ | *R. sphaeroides* | 1M56 | 2.3 | wild-type enzyme |
| | | 1M57 | 3.0 | EQ(I-286) mutant enzyme |
| cytochrome $aa_3$ | bovine heart | 2OCC | 2.3 | oxidized state |
| | mitochondria | 1OCR | 2.35 | reduced state |
| | | 1V54 | 108 | oxidized state |

*Note. The Protein Data Bank can be found at www.rcsb.org.

these problems by binding $O_2$ to the heme $a_3$ iron in the high-spin state and by providing a second electron donor, $Cu_B$, in the immediate vicinity. The rates at which bacterial oxidases catalyze the $O_2$ reduction are remarkably rapid with turnover numbers of up to several hundred $O_2$ molecules per second. The rapid uptake of $O_2$ may be facilitated by hydrophobic channels leading from the membrane core to the catalytic site, as suggested from analyses of the crystal structures of heme–copper oxidases.

The mechanism of the $O_2$-reduction has been primarily investigated in detail in cytochromes $aa_3$ from *R. sphaeroides*, *P. denitrificans*, the closely related mitochondrial enzyme, as well as in cytochrome $bo_3$ from *E. coli*. The reaction sequence is most likely the same for all oxidases, but the mechanism described below has been identified primarily from studies of these enzymes.

Initially, the electrons are transferred one at a time to the binuclear center. When both heme $a_3$ and $Cu_B$ are reduced (i.e., $Fe^{2+}_{a_3}$ and $Cu^{+}_{B}$, state **R** in Figure 2), $O_2$ binds to heme $a_3$ (the state is called **A**). After binding of $O_2$ the O–O bond is cleaved, which results in formation of a ferryl group ($Fe^{4+}_{a_3}{=}O$, see Figure 2), where one electron and a proton are donated by an internal group, presumably a highly conserved Tyr at the catalytic site, which would form a radical. Even though the binuclear center is in a two-electron reduced state, formally four electrons are donated to $O_2$ in this reaction because two electrons are donated by $Fe_{a_3}$($Fe^{2+}_{a_3} \rightarrow Fe^{4+}_{a_3}$), one by $Cu_B$($Cu^{+}_{B} \rightarrow Cu^{2+}_{B}$ and one by the Tyr. For historical reasons, this state is called "peroxy" and denoted **P**.

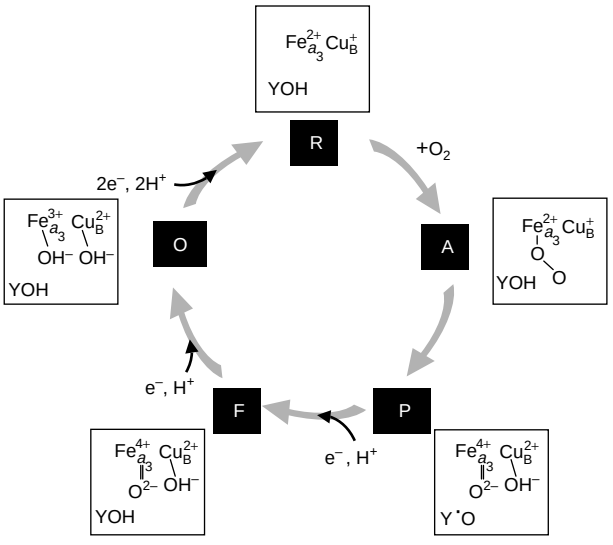



**FIGURE 2** The reaction cycle of cytochrome *c* oxidase. State **O** is the oxidized enzyme. The two-electron-reduced catalytic site (state **R**) binds $O_2$ (forming state **A**). In the first step of $O_2$ reduction, a ferryl state is formed (**P**), where one electron and one proton are transferred from a Tyr residue at the catalytic site. The transfer of the third electron to the catalytic site is accompanied by proton uptake from the bulk *N*-side solution and the formation of state **F**. In the next step, the fourth electron is transferred to the binuclear center associated with proton uptake from the bulk *N*-side solution.

The transfer of the third electron to the binuclear center is associated with proton uptake from the inside bulk solution. The intermediate that is formed is called "ferryl" and is denoted **F** (Figure 2). It is assumed to have the same chemical structure as **P**, except for the additional electron and proton at the binuclear center, which are presumably transferred to the Tyr radical. The transfer of the next, forth electron to the binuclear center is also associated with proton uptake from the inside bulk solution and results in formation of the oxidized binuclear center, denoted **O**.

## Proton Pumping

In 1977, Mårten Wikström showed that in addition to the charge separation associated with the $O_2$ reduction reaction, cytochrome *c* oxidase also releases protons to the *P*-side of the membrane. In other words, the enzyme is a proton pump, which translocates one proton from the *N*-side to the *P*-side of the membrane per electron transferred to $O_2$. Consequently, since there is no proton carrier in the enzyme that can bodily move protons across the membrane, there must be a specific mechanism by which the enzyme uses the free energy from reduction of $O_2$ to translocate protons across the membrane. This function must be reflected in specific structural elements of the molecular machine. The molecular mechanism by which heme–copper oxidases pump protons is not known. The general view is that during the process the enzyme must provide an alternating access of protons to the two sides of the membrane and have this alteration strictly coupled to specific transitions of the catalytic cycle. Such changes in the accessibility for protons to the two sides of the membrane are likely to be achieved by breakage and formation of hydrogen bonds in the proton-transfer pathways, for example, through local rearrangements of amino-acid residue side chains. It has been shown that the **R** → **P** transition is not associated with proton pumping, while the **P** → **F** and **F** → **O** transitions are associated with the translocation of one proton in each step. The definite assignment of other reaction steps in which proton translocation occurs and conditions under which this takes place is at present not definitely settled.

As indicated above, in the initial step of the reaction, the $O_2$ molecule is formally reduced by four electrons (to form state **P**, Figure 2). An advantage of such a four-electron reduction of $O_2$ in a single step is that it prevents accumulation of potentially harmful, partly reduced $O_2$ intermediates. However, since the formation of **P** is not associated with proton translocation, this mechanism

requires that the free energy available from the $O_2$--reduction reaction is conserved within the enzyme, for example by the formation of high-p$K_a$ internal proton acceptors; and the free energy can be used for proton translocation in the following steps of the reaction.

## Some Experimental Techniques Used to Study Heme–Copper Oxidases

Present knowledge about the $O_2$-reduction mechanism is derived from the use of a large number of experimental techniques. One of these is the "flow-flash" technique, pioneered by Quentin Gibson and Colin Greenwood. The enzyme reduced with 2–4 reduction equivalents and with CO bound to heme $a_3$ is mixed with an $O_2$ solution after which the CO ligand is flashed off by means of pulsed illumination, which initiates the reaction of the enzyme with $O_2$. The application of this technique has made it possible to observe, with microsecond time resolution, the transitions between different states. The progress of the reaction is followed, e.g., by observing absorbance changes of the redox sites, by measuring voltage changes that originate from the movement of charges in enzyme reconstituted in lipid vesicles, or by using time-resolved resonance-Raman spectroscopy to obtain information about the structure of the oxygen intermediates. In addition, Fourier Transform Infrared Difference spectroscopy is used to identify specific residues that undergo changes in their protonation state and/or hydrogen-bonding patterns, and Electron Paramagnetic Resonance spectroscopy is used to explore the electronic configuration and the chemical environment of the redox centers. The use of the techniques discussed above, combined with the use of site-directed mutagenesis, has provided important insights into the catalytic mechanism.

### See Also the Following Articles

Cytochrome *c* • Heme Proteins • P-Type Pumps: Copper Pump

### Glossary

**aerobe** Organism that uses oxygen ($O_2$) as the terminal electron acceptor in respiration.

**cytochrome** A heme protein serving as an electron carrier.

**proton electrochemical gradient** Sum of the contributions of the proton concentration difference (pH) and voltage across a membrane (in units of energy, e.g., kJ/mol or eV).

**proton pump** Integral membrane protein that translocates protons across the membrane without the use of mobile proton carriers (which would bodily carry protons across the membrane, e.g., a quinone).

**proton-transfer pathway** Typically, an arrangement of protein-bound water molecules and hydrophilic or protonatable amino-acid residues used for the transfer of a proton from a donor to an acceptor (where one of these may be the bulk solution).

**redox-active cofactor or redox site** Group within a protein (e.g., a metal ion) that can accept and give electrons in its oxidized and reduced states, respectively.

### Further Reading

Abramson, J., Riistama, S., Larsson, G., Jasaitis, A., Svensson-Ek, M., Laakkonen, L., Puustinen, A., Iwata, S., and Wikström, M. (2000). The structure of the ubiquinol oxidase from *Escherichia coli* and its ubiquinone binding site. *Nature Str. Biol.* **7**, 910–917.

Brzezinski, P., Larsson, G., and Ädelroth, P. (2003). Functional aspects of heme–copper terminal oxidases. In *Advances in Photosynthesis and Respiration* (D. Zannoni, ed.) Kluwer Academic, Dordrecht/ Norwell, MA.

Ferguson-Miller, S., and Babcock, G. T. (1996). Heme/copper terminal oxidases. *Chem. Rev.* **96**, 2889–2907.

Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995). Structure at 2.8 Å resolution of cytochrome c oxidase from Paracoccus denitrificans. *Nature* **376**, 660–669.

Soulimane, T., Buse, G., Bourenkov, G. P., Bartunik, H. D., Huber, R., and Than, M. E. (2000). Structure and mechanism of the aberrant $ba_3$-cytochrome *c* oxidase from *Thermus thermophilus*. *EMBO. J.* **19**, 1766–1776.

Svensson-Ek, M., Abramson, J., Larsson, G., Törnroth, S., Brzezinski, P., and Iwata, S. (2002). The X-ray crystal structures of wild-type and EQ(I-286) mutant cytochrome *c* oxidases from *Rhodobacter sphaeroides*. *J. Mol. Biol.* **321**, 329–339.

Verkhovsky, M. I., Jasaitis, A., Verkhovskaya, M. L., Morgan, J. E., and Wikström, M. (1999). Proton translocation by cytochrome *c* oxidase. *Nature* **400**, 480–483.

Wikström, M. K. F. (1977). Proton pump coupled to cytochrome c oxidase in mitochondria. *Nature* **266**, 271–273.

Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998). Redox-coupled crystal structural changes in bovine heart cytochrome *c* oxidase. *Science* **280**, 1723–1729.

Zaslavsky, D., and Gennis, R. B. (2000). Proton pumping by cytochrome *c* oxidase: Progress, problems and postulates. *Biochim. Biophys. Acta* **1458**, 164–179.

### Biography

Peter Brzezinski is Professor of Biochemistry at the Department of Biochemistry and Biophysics at Stockholm University in Stockholm, Sweden. He specializes in the field of bioenergetics, and his principal research interests are mechanisms of electron- and proton-transfer reactions in biological systems, in particular in membrane-bound proteins. He holds a Ph.D. in physics from Chalmers University of Technology in Göteborg, Sweden and received his postdoctoral training at the University of California, San Diego.

# Cytochrome P-450

**Rita Bernhardt**
*Saarland University, Saarbrücken, Germany*

**Cytochromes P-450 are ubiquitously distributed multicatalysts, which were discovered approximately 50 years ago. They possess a high degree of complexity and display a broad field of activity. Although already more than 2500 different P450 forms have been described, new forms are constantly being found, opening up new research fields. They are the key enzymes responsible for the metabolism of many drugs, carcinogens, alkaloids, pesticides, and other important xenobiotics. Moreover, they are involved in a variety of physiological processes such as steroid hormone, eicosanoid, vitamin D, and bile acid biosynthesis. Cytochromes P-450 are hemoproteins that use molecular oxygen to catalyze various reactions. They get the electrons necessary for oxygen activation and substrate hydroxylation from NADH or NADPH, in general via the action of electron-transferring proteins. Most cytochromes P-450 are membrane-bound. Many of them are inducible, but some are constitutively expressed. Defects in some of the cytochromes P-450 lead to pathological effects, such as congenital adrenal hyperplasia and hypertension, and adverse drug effects.**

## Cytochrome P-450—General Aspects

There are two general classes of enzymes involved in oxygen metabolism: oxidases, transferring electrons from a substrate to oxygen, and oxygenases, transferring oxygen to a substrate after reductive splitting of molecular oxygen. Oxygenases can be divided into dioxygenases and monooxygenases. Monooxygenases (mixed-function oxidases) catalyze the incorporation of a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water. The monooxygenases are divided into two classes, the internal and the external monooxygenases. Internal monooxygenases extract two reducing equivalents from the substrate to reduce one atom of dioxygen to water, whereas external monooxygenases use an external reductant. Cytochromes P-450 are external monooxygenases. Initially the microsomal drug and xenobiotic-metabolizing enzymes were referred to as mixed-function oxidases, but in more recent years the term monooxygenase has become the more accepted one.

Cytochromes P-450 got their name from their character as a hemoprotein and from their unusual spectral properties, displaying a typical absorption maximum of the reduced CO-bound complex at 450 nm (Figure 1)—cytochrome stands for a hemoprotein, P for pigment, and 450 reflects the absorption peak of the CO complex at 450 nm. The ability of reduced P450 to produce an absorption peak at 450 nm upon CO binding is still used for the estimation of P450 content. The red shift of approximately 30 nm observed in cytochromes P-450 means that the distribution of electron density at the heme is significantly perturbed compared to other cytochromes. It has been documented that it is the thiolate sulfur that causes this effect by means of a direct bond to the iron. The Soret band (named after its discoverer) describes the absorption band of hemoproteins at approximately 380–420 nm.

Cytochrome P-450 systems catalyze the following reaction:

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$$

They catalyze reactions as diverse as hydroxylation; N-, O-, and S-dealkylation; sulfoxidation; epoxidation; deamination; desulfuration; dehalogenation; peroxidation; and N-oxide reduction. Their substrates include fatty acids, steroids, and prostaglandins, as well as a multitude of foreign compounds such as drugs, anesthetics, organic solvents, ethanol, alkylaryl hydrocarbon products, pesticides, and carcinogens. This diversity of catalyzed reactions and acceptable substrates has attracted researchers from diverse fields to the study of cytochrome P-450 systems (Figure 2). In addition to pharmacologists and toxicologists, endocrinologists, physiologists, microbiologists, organic chemists, plant biologists, and environmental scientists also are working on diverse aspects of P450 function and regulation.

It is obvious that this diversity of substrates and catalyzed reactions cannot be managed by only a few different isoforms. In the human genome alone 57 different P450 genes have been found, and in total more than 3000 different isoforms have been characterized

 © 2004, Elsevier Inc. All Rights Reserved. 

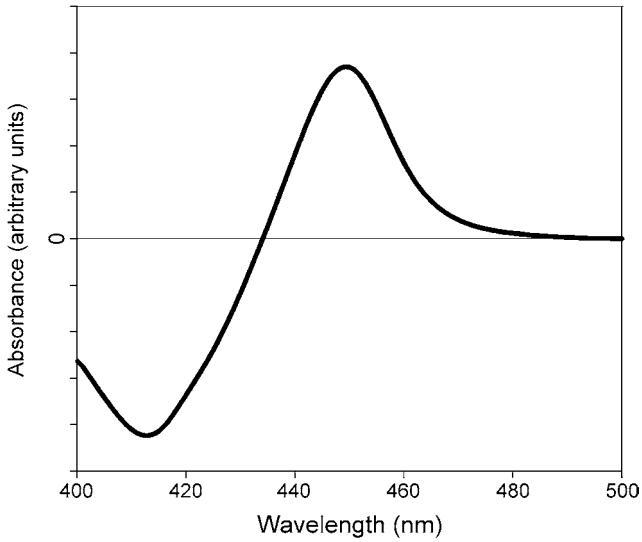



**FIGURE 1** CO-difference spectrum of reduced versus oxidized cytochrome P-450 (CYP106A2).

(D. Nelson lists the available P450 sequences identified so far on his website). This superfamily of proteins (and their respective genes) is divided into families, subfamilies, and finally into individual members according to similarities in primary structure. A new nomenclature has been introduced in which CYP and a series of numbers and letters are used to characterize each P450 as a hemoprotein. For example, in CYP1A1, for cytochrome P4501A1 (previously called P450c), the first arabic number defines the gene family, the following letter defines the subfamily, and the second number defines the individual enzyme. Members of the same gene family are defined as usually having $<40\%$ sequence identity to a P450 protein from any other family. Mammalian sequences within the same subfamily are always $>55\%$ identical. The numbers of individual P450 enzymes in different species differ, the highest numbers observed so far being in plants.

## Structural Organization of Cytochrome P-450 Systems

As already mentioned, cytochromes P-450 belong to the external monooxygenases. This means that they need an external electron donor to transfer the electrons necessary for oxygen activation and the following substrate hydroxylation. Two main classes of cytochromes P-450 can be defined with respect to their electron-supporting system, although other subclasses also occur: the microsomal type and the mitochondrial/bacterial type (Figure 3).

Microsomal cytochromes P-450 are membrane-bound and accept electrons from a microsomal NADPH–cytochrome P-450 reductase, containing flavin adenine dinucleotide (FAD) and falvin mononucleotide (FMN). All drug- and xenobiotica-metabolizing cytochromes P-450 isolated so far belong to this class. In addition, CYP102 (P450BM-3) isolated from *Bacillus megaterium* was shown to belong to this class. This P450 system consists of a polypeptide chain with two different domains, one containing the hemoprotein and the other containing a flavoprotein with FAD and FMN. Most of the other bacterial cytochromes P-450 belong to

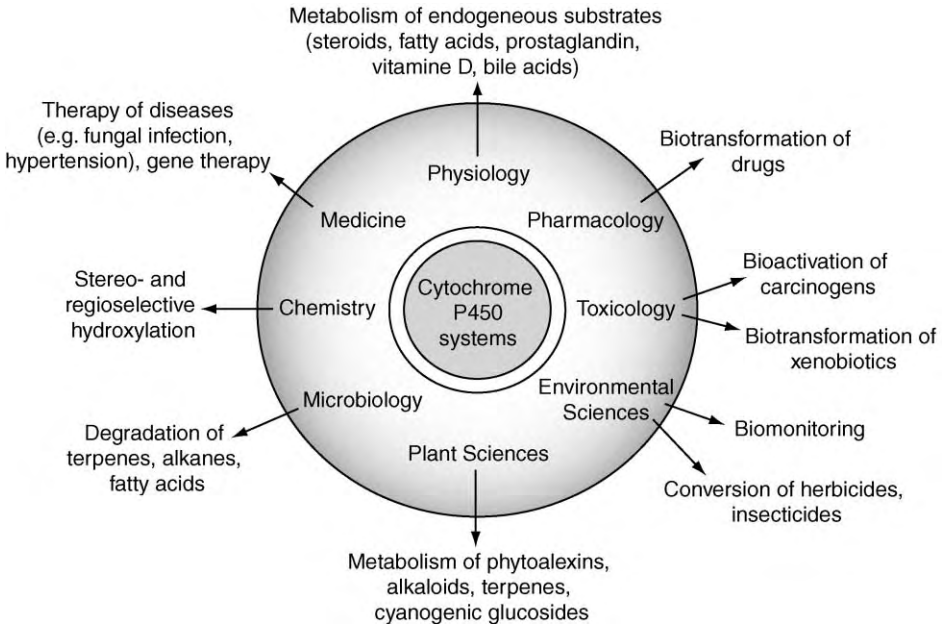



**FIGURE 2** Cytochrome P-450 research and application fields.

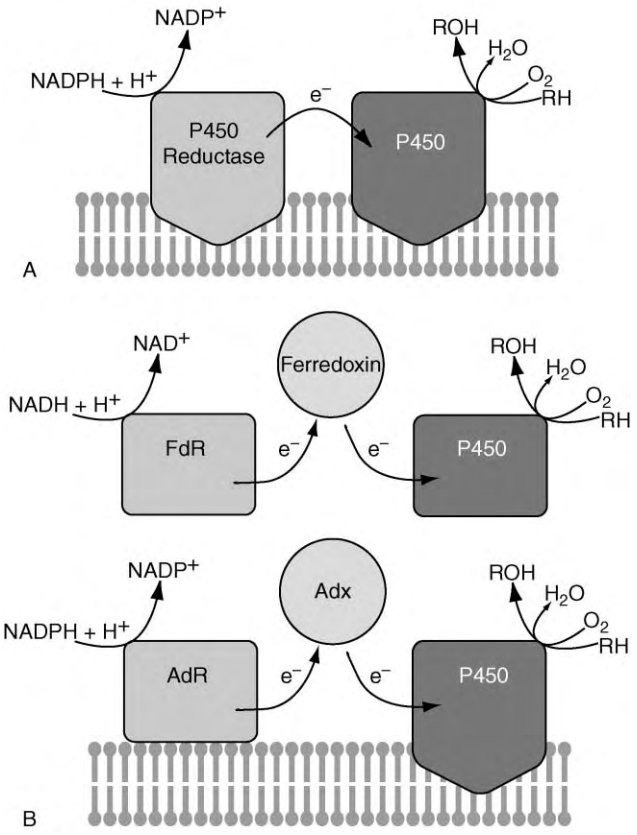



**FIGURE 3** Model for the organization of cytochrome P-450 systems. (A) Microsomal type. (B) Mitochondrial/bacterial type.

the second class. They are soluble and obtain the electrons necessary for the reaction mechanism from an NADH-dependent FAD-containing reductase via an iron–sulfur protein of the [2Fe–2S] type.

Mitochondrial cytochromes P450, which are involved e.g., in the side-chain cleavage of cholesterol, the 11β-hydroxylation of deoxycortisol, and the production of aldosterone, also belong to the second class. These cytochromes P-450 are localized in the inner mitochondrial membrane, where the [2Fe–2S] protein, called adrenodoxin in the case of adrenal steroid hydroxylase systems, is a soluble protein of the matrix. The FAD-containing reductase, adrenodoxin reductase, is associated with the inner mitochondrial membrane.

The interaction of the cytochromes P-450 with their corresponding electron donors is a necessary prerequisite of the catalytic cycle. Its specificity guarantees a sufficient reaction rate of catalysis and likewise a discrimination between the different potential donors and acceptors of electrons to protect the system from shunt reactions.

Because many different isoenzymes in liver microsomes have to interact with only one type of reductase, the binding site for reductase is very similar or identical on various cytochromes P450. Salt bridges are responsible for the recognition of reductase by the cytochromes P-450 and for the correct orientation of the proteins to one another. In addition to microsomal reductase, some microsomal cytochromes P-450 are able to accept the second electron from cytochrome $b_5$. cytochrome $b_5$ has also been shown to exert a differential stimulatory action, depending on the form of cytochrome P-450 and the substrate metabolized.

In mitochondrial steroid hydroxylases and in the camphor hydroxylating bacterial cytochrome P-450 (CYP101) system, a charge–pair interaction mechanism has been demonstrated by chemical modification, site-directed mutagenesis studies, and structural data of electron-transfer complexes. Like microsomal reductase, the mitochondrial ferredoxin has been shown to deliver electrons to different cytochromes P450. From the available data, a shuttle model is favored, in which the oxidized ferredoxin interacts first with the ferredoxin reductase to undergo reduction, with the formation of a $Fe^{3+}$–$Fe^{2+}$ iron–sulfur cluster. It dissociates from the reductase and then interacts with the respective cytochrome P450, to which it delivers this electron before going back to the reductase, and transfers the second electron to the cytochrome P450. The mechanism of electron transfer between the components of the different cytochrome P-450 systems, one of the fundamental problems in life sciences, is not well understood.

## Reaction Cycle

The generally accepted mechanism of cytochrome P450-dependent substrate conversion is depicted in the overall scheme presented in Figure 4. The first step of the reaction cycle is the formation of the substrate–enzyme complex. Substrate binding induces structural changes in the cytochrome P-450 that may result not only in a

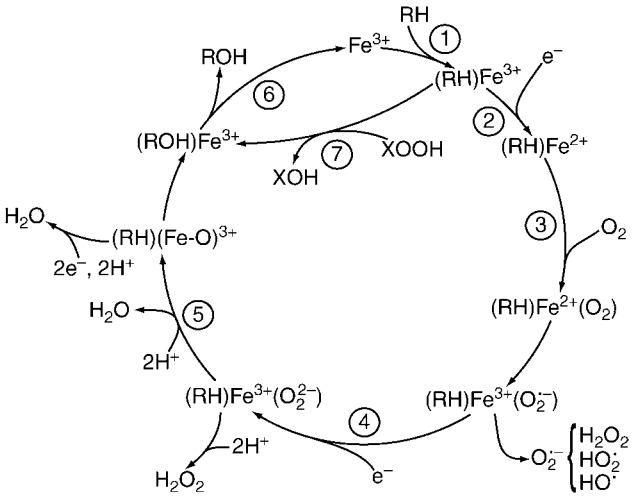



**FIGURE 4** Reaction cycle of cytochromes P450.

spin shift, but also in changes of the redox potential and in changed binding affinities between interacting components of the cytochrome P-450 system. The second step of the reaction cycle is the introduction of the first electron, either by NADPH-dependent reductase or via a ferredoxin (see Figure 3). Iron (3+) is reduced to iron (2+). Although in some cytochromes P450 the substrate binding is a prerequisite for electron transfer, this is not a universal requirement. In the third step of the reaction cycle, the hemoprotein reduced by one electron binds the dioxygen molecule. As shown in Figure 4, from this complex a superoxide anion radical can be released. Step 4 of the reaction cycle is the introduction of the second electron. In some instances, another microsomal hemoprotein, cytochrome $b_5$, can facilitate catalysis by providing the second electron. From the $(RH)Fe^{3+}(O_2^{2-})$ complex, hydrogen peroxide can be split off. The structure of the activated oxygen complex and the precise mechanism of oxygen cleavage are not fully understood yet. Step 5 in the reaction cycle is the removal of the terminal oxygen atom from the dioxygen ligand, that is, the cleavage of the dioxygen bond. Heterolysis of the oxygen–oxygen bond also results in the formation of a putative oxyferryl species. Heterolytic cleavage in P450cam (CYP101), where most of the mechanistic studies have been performed, was shown to be facilitated by hydrogen bonding of the ferric hydroperoxide via a water molecule to a highly conserved threonine residue (Thr252 in CYP101), which is involved in a proton transfer network that promotes oxygen cleavage. The introduction of two electrons and two protons to the putative oxyferryl species may lead to the formation of water. Finally, in step 6 the hydroxylated product dissociates and the cycle can start again. Interestingly, in many but not all cytochromes P450 a shunt reaction can proceed (step 7), in which the substrate can be hydroxylated directly by peroxides such as hydrogen peroxide, cumene hydroperoxide, and *tert*-butyl hydroperoxide without the necessity of an interaction with an electron-donating system. Taken together, cytochromes P-450 do not catalyze just monooxygenase, but also oxidase and peroxidase reactions. Variations of this scheme for the reaction mechanism of cytochromes P-450 occur with different cytochrome P-450 systems such as thromboxane and prostacyclin synthase, nitric oxide reductase (CYP55A1), and others.

## Regulation of Cytochrome P-450 Systems

The regulation of enzyme systems is possible at different levels. When considering cytochrome P-450 systems, the complexity of the reaction cycle, the organization within membrane systems for most of the cytochromes P450, and their organ- and tissue-specific expression imply various possible ways for regulatory mechanisms to work. Interaction between these mechanisms then leads to the tuned response of these enzyme systems to endogenous and exogenous signals in terms of acute and long-term reactivities of cytochromes P450.

First it is possible to regulate cytochromes P-450 on the level of the organism. Some of the cytochromes P-450 are expressed in age-, tissue-, and sex-dependent manners. These differences in the expression pattern are governed by sex or other hormones. There are also clearly developmental influences on the expression and function of various cytochrome P-450 systems.

Cellular regulation of cytochromes P-450 is extremely complex and can occur on at least two different levels, enzyme induction and post-translational modification of enzymes. The induction of drug-metabolizing enzymes was noted in the early 1950s. Since then specific inducers for various cytochrome P-450 families and subfamilies have been identified. It can be demonstrated that the inductor functions by enhancing the rate of mRNA synthesis of a special cytochrome P450. In some cases, receptors (the best-studied system currently is the aryl hydrocarbon, Ah, receptor) are involved in mediating the effect of the inducer. Not all cytochromes P450 are, however, inducible; some of them are constitutively expressed.

After the biosynthesis of cytochromes P450, the enzymes might be subject to post-translational modifications to exert a short-term control of activity. One of the most common types of post-translational modifications in eukaryotic organisms is protein phosphorylation, which has been demonstrated for several steroid hydroxylases such as the microsomal CYP7 and the mitochondrial CYP11A1 and also for the drug-metabolizing microsomal CYP2B4.

Because nearly all the cytochromes P-450 are bound to membranes, they can be regulated also at that level. Exclusions are most of the bacterial cytochromes P450, such as CYP101, CYP102, and CYP108, which are soluble. The composition of the membrane (e.g., protein–lipid as well as lipid-mediated protein–protein interactions) exert a functional control on cytochromes P450. Lipids appear to function in at least three ways—they (1) stabilize and induce a functionally active conformation of cytochromes P-450 and the corresponding electron-transfer systems, (2) modulate the electron transfer, and (3) mediate interactions between cytochromes P-450 and the electron-donor systems.

Finally, cytochromes P-450 can be regulated at the molecular level. This includes regulation of the activities by changes of the primary sequence (e.g., by polymorphisms), the spin equilibrium, protein–electron donor interactions, and protein–protein interactions.

## Important Functions of Cytochrome P-450 Systems

### Cytochromes P-450 and Drug Metabolism

Cytochromes P-450 are able to perform the biotransformation of an enormous variety of substrates. Taking into account all drugs metabolized by enzymes, cytochromes P-450 are responsible for more than two-thirds of these reactions. The most important cytochrome P-450 form for biotransformation in humans is CYP3A4; it is involved in the metabolism of more than one-half of the known drugs and xenobiotics, such as nifedipine, cyclosporin, erythromycin, gestodene, and aflatoxins. This variety of substrates makes CYP3A4 one of the most important enzymes for drug metabolism. The fact that a single cytochrome P-450 is responsible for the metabolism of many different drugs may lead to competition among various drugs for the same enzyme and thus to drug–drug interactions, resulting in higher plasma levels of the less successful (i.e., displaying a lower affinity for the particular cytochrome P450) substrate and even to fatal side effects of the drug treatment.

Another fact complicating drug treatment is the presence of genetic polymorphisms in some of the drug-metabolizing enzymes. It has been shown that 5–10% of Caucasians suffer from a decreased ability to metabolize certain drugs such as debrisoquine and bufuralol (i.e., they are poor metabolizers), due to the presence of nonfunctional alleles of CYP2D6 in these individuals. A smaller portion of patients metabolize the corresponding drugs extremely rapidly (i.e., they are extensive metabolizers), due to having multiple copies of this gene. These pharmacogenetic effects are being studied in many laboratories and will certainly lead to personalized pharmacotherapy.

### Physiological Role of Cytochromes P450

In addition to being involved in drug metabolism and xenobiotica degradation, cytochromes P-450 also play a pivotal role in various physiological processes in humans. They are central in the biosynthesis of steroid hormones (sex hormones, glucocorticoids, and mineralocorticoids), vitamin D, fatty acids, bile acids, and eicosanoids. Some severe defects are connected to mutations in cytochrome P-450 genes, such as congenital adrenal hyperplasia, which is mostly due to a steroid 21-hydroxylase (CYP21) deficiency. In addition, human essential hypertension can be caused by the overproduction of aldosterone produced by CYP11B2. More recently, products of cytochrome P-450 reactions such as oxysterols and the endothelium-derived hyperpolarizing factor (EDHF) have been found to play important roles in cellular cholesterol homeostasis, inhibition of cellular proliferation, gene regulation, endothelium-dependent dilation, and enhanced endothelial cell proliferation.

### Cytochrome P-450 Diversity

Cytochromes P-450 are found in all kingdoms—eubacteria, archaebacteria, fungi, plants, fish, insects, and vertebrates. More than 250 different forms have been found in the plant *Arabidopsis thaliana*. It was shown that plant cytochromes P450 play a role in the metabolism of a variety of secondary metabolites, in plant–insect interaction, in herbicide metabolism, and in other vital functions. Due to the diversity of catalyzed reactions it can be anticipated that plant cytochromes P450 will offer a broad field for future applications.

### See Also the Following Articles

Oxygenases • Vitamin D

### Glossary

**CYP** Nomenclature for the enzymes of the cytochrome P-450 superfamily.

**cytochrome P-450** Hemoprotein showing an unusual absorption of the reduced CO complex at 450 nm.

**genetic polymorphism** Inherited deficiencies of single enzymes such as CYP2D6 and CYP2C19.

**glucocorticoids** Steroid hormones regulating glucose metabolism, regulating the stress response of the body, and suppressing inflammation.

**hormone** Chemical messenger secreted into the circulating blood.

**mineralocorticoids** Steroid hormones regulating the salt and water levels of the body and in this way the blood pressure.

### Further Reading

Bernhardt, R. (1996). Cytochrome P450: Structure, function, and generation of reactive oxygen species. *Rev. Physiol. Biochem. Pharmacol.* **127**, 137–221.

Bureik, M., Lisurek, M., and Bernhardt, R. (2002). The human steroid hydroxylases CYP11B1 and CYP11B2. *Biol. Chem.* **383**, 1537–1551.

Guengerich, F. P. (2002). Update information on human P450s. *Drug Metab. Rev.* **34**, 7–15.

Guengerich, F. P., Parikh, A., Yun, C. H., Kim, D., Nakamura, K., Notley, L. M., and Gillam, E. M. (2000). What makes P450s work? Searches for answers with known and new P450s. *Drug Metab. Rev.* **32**, 267–281.

Meyer, U. A. (2000). Pharmacogenetics and adverse drug reactions. *Lancet* **356**, 1667–1671.

Nelson, D. Nelson Lab Homepage. Available at: http://drnelson.utmem.edu/nelsonhomepage.html.

Ruckpaul, K., and Rein, H. (eds.) (1989–1994). *Frontiers in Biotransformation*, Vol. 1–9, Akademie-Verlag, Berlin.

## Biography

Rita Bernhardt is a Full Professor at the Saarland University and Head of the Institute of Biochemistry. Her principal research interests are cytochrome P-450 systems. She holds a Ph.D. from Moscow State Lomonossov University and received her postdoctoral training at the Central Institute of Molecular Biology of the Academy of Sciences of the GDR in Berlin–Buch, the University of Illinois at Urbana-Champaign, and the University of Sendai, Japan. She uses a variety of biochemical, biophysical, and engineering techniques to study cytochrome P-450 system functions and improve their catalytic efficiency and stability, with special attention to the structure, function, and regulation of mitochondrial steroid hydroxylase systems.

# Cytokines

**Andrea L. Wurster and Michael J. Grusby**
*Harvard School of Public Health, Boston, Massachusetts, USA*

**Cytokines are a diverse set of small, secreted proteins that help direct many critical aspects of an immune response. Cytokines are rapidly produced in response to foreign antigen exposure and can promote the expansion, activation, recruitment, and differentiation of the responding cell types. However, cytokine expression and signaling must be tightly regulated because dysregulated cytokine responses can lead to pathological conditions such as autoimmunity and asthma.**

## General Features of Cytokines

### Nomenclature

Cytokines have been referred to by a number of labels depending on the cell types that produce them or their functional properties. For example, cytokines that are derived primarily from mononuclear cells such as macrophages have been referred to as monokines while the cytokines produced by activated T lymphocytes are termed lymphokines. Cytokines that specifically regulate the migration of other cells are called chemokines in reference to their chemotactic properties. Historically cytokines were also referred to as interleukins since in a general sense they are being produced by and acting on leukocytes. It is the term interleukin that spawned a universal numerical nomenclature for cataloguing newly identified and characterized cytokines (i.e., IL-4). Sequence comparisons between previously identified cytokines and the recently completed mouse and human genome databases have resulted in a rapid increase in the discovery of new cytokines with the most recently reported being IL-29.

### Pleotropism and Redundancy

With so many cytokines now known to exist, an added complexity to their biology is that most cytokines are pleiotropic. This refers to the notion that cytokines do not just act on one particular cell type but can have wide effects on a number of cell types within the immune system as well as on cell types outside of the immune system. A clinically relevant example is the cytokine IL-13. IL-13 is a T cell derived cytokine whose effects on B lymphocytes and monocytes (up-regulation of MHC class II and inhibition of inflammatory cytokine production) have been well described. However, IL-13 has also been shown to be critically involved in the pathogenesis of allergic asthma and is thought to mediate its effects by its actions on epithelial and smooth muscle cells in the lung.

Cytokines are also redundant in nature. This means that many (but not all) biological properties originally described for one cytokine can also be ascribed to others. For instance, when IL-2 was originally discovered it was thought to be the primary T cell growth hormone. Subsequently it has been found that a number of other cytokines can also promote T cell expansion, including IL-4. This redundancy is borne out in genetic knockout experiments of individual cytokines often resulting in mice with subtle defects in their immune responses suggesting that the loss of a particular cytokine can be compensated for by the action of another.

### Regulated Expression

The expression of cytokines is also highly regulated. Cytokine gene transcription usually occurs rapidly after an inducing stimulus. The rapid increase in cytokine gene mRNA results in a burst of cytokine protein secretion into the surrounding milieu and can act directly on the cell that produced it or on neighboring cells. For example, T lymphocytes produce large amounts of IL-2 within minutes of encountering antigenic peptides which functions to promote their own expansion. Likewise, when a phagocytic monocyte encounters bacterial lipids it rapidly produces the cytokine TNF resulting in recruitment and activation of additional inflammatory leukocytes. This rapid increase in transcription is usually transient and is extinguished rapidly resulting in a self-limited event. The transitory nature of the cytokine response is imperative since, in the case of TNF, high levels and sustained cytokine production can cause systemic problems such as cachexia and septic shock.

Cytokine expression is not only highly regulated temporally but can also be restricted in a cell type specific manner. For example, CD4+ T helper cells can be divided into two distinct subsets based precisely on

 © 2004, Elsevier Inc. All Rights Reserved. 

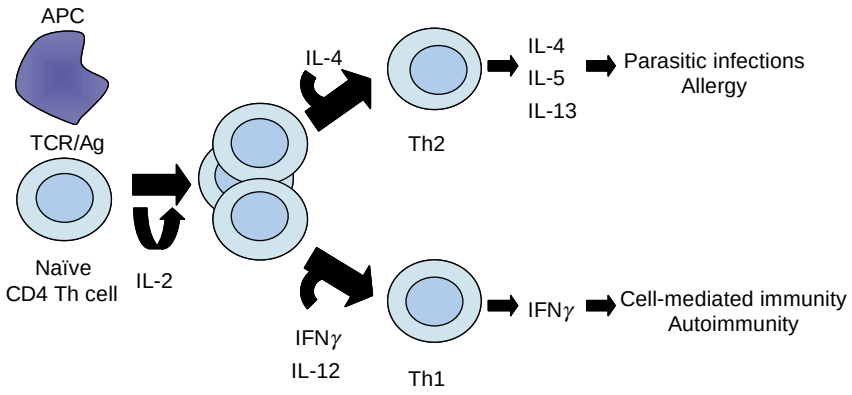



**FIGURE 1** T helper cell differentiation. Th cells can differentiate into functionally different subsets depending on the cytokines they are exposed to during antigen activation. Developing Th cells that are exposed to IL-4 differentiate into Th2 cells capable of producing IL-4, IL-5, and IL-13. Th2 cells are important for the clearance of parasitic infections but can also contribute to the pathogenesis of asthma. Th cells that develop in the presence of IFN$\gamma$ and IL-12 ultimately become IFN$\gamma$-producing Th1 cells and play an important role in the clearance of intracellular bacteria but can also contribute to the development of autoimmunity.

their cytokine expression profiles (Figure 1). These individual subsets carry out very different roles in an immune response in part due to the cytokines they produce. T helper type 1(Th1) cells are characterized by the production IFN$\gamma$, a cytokine involved in the activation of macrophages to kill phagocytosed microbes. Not surprisingly, Th1 cells are heavily involved in the clearance of intracellular bacteria. Alternatively, T helper type 2 (Th2) cells are characterized by their production of IL-4, IL-5, and IL-13 which directly promote the production of the IgE antibody isotype and stimulate eosinophils. These downstream responses are critical for the clearance of helminthic parasites.

## The Role of Cytokines in Immune Responses

It is clear from the discussion above that cytokines can promote a variety of effects on a number of cell types resulting in a wide range of outcomes. Ultimately, the specific expression of and response to cytokines helps orchestrate a tightly regulated immune response.

### Cytokines and the Innate Immune Response

Innate immunity is characterized by the initial recognition that a foreign pathogen, such as a virus or bacteria, is in the host. Cytokines play an important role in alerting and activating the immune system to an infection.

#### Cytokine Response to Bacterial Infection

The cytokine TNF plays an important role in the initial inflammatory response to bacteria especially in the recruitment of leukocytes to the site of infection (Figure 2). When a mononuclear phagocyte, such as a macrophage, encounters gram-negative bacteria it recognizes a product of the cell wall called LPS. LPS binds to a cell surface receptor on the macrophage called CD14 and stimulates the rapid production of TNF. The local presence of TNF serves to recruit other cells, neutrophils and monocytes, to the site of infection. TNF does this by stimulating the expression of adhesion molecules on the surrounding endothelial cells, which capture the leukocytes as they pass by the activated endothelium. TNF also stimulates the production of chemokines from the activated endothelial cells, which further serves to increase recruitment and activation of leukocytes and ultimately results in their transmigration through the vessel wall directly to the infection site. Another cytokine, IL-1, is also produced by macrophages after bacterial exposure and promotes similar inflammatory responses.

Macrophages also respond to bacterial products by producing IL-12 (Figure 2). IL-12 is an important activator of IFN$\gamma$ production from NK cells and T cells. This IFN$\gamma$ then feeds back onto the macrophages by enhancing their microbicidal functions to destroy phagocytosed bacteria. IL-12 also directly increases the cytolytic activity of NK cells themselves. Mice made genetically deficient for functional IL-12 have defects in their IFN$\gamma$ production and NK cell function.

#### Cytokine Response to Viral Infection

The cytokines IFN-$\alpha$ and IFN-$\beta$, also known as type I IFNs, are important mediators of the innate immune

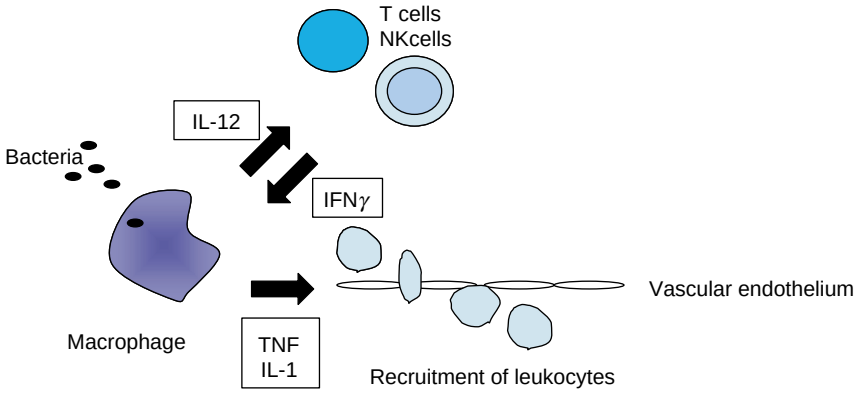



**FIGURE 2** Cytokine response to a bacterial infection. Macrophage activation by exposure to bacterial products results in TNF and IL-1 secretion. TNF and IL-1 activate nearby vascular endothelial cells to permit recruitment of leukocytes to the infection site. Activated macrophages also produce IL-12 leading to increased IFNγ production from NK and T cells. IFNγ feeds back on the macrophage to increase the phagocytic potential and microbicidal properties of the macrophage.

response to a viral infection. When a cell is infected with a virus, or exposed to double-stranded RNA molecules that mimic viral replication, production of type I IFNs is dramatically induced. The expression of type I IFNs has a number of consequences in the control of a viral infection. First, exposure to type I IFNs induces the expression of enzymes that directly interfere with viral replication and this helps to protect neighboring cells from becoming infected. Also, type I IFNs act to increase class I MHC expression ultimately resulting in enhanced CTL responses to infected cells. Additionally, type I IFNs directly boost the lytic activity of NK cells.

## Cytokines and the Adaptive Immune Response

The adaptive immune response is characterized by the specific activation and expansion of antigen-specific lymphocytes in response to a pathogen as well as immunological memory. Cytokines play a critical role in the proliferation of activated lymphocytes and promoting their differentiation into distinct effector subsets and the induction of memory. Here are just a few examples.

### IL-2

As mentioned above, IL-2 has been described chiefly as a T cell growth factor, although it is now recognized that IL-2 plays important roles in several aspects of the adaptive immune response. When a T cell recognizes foreign peptide antigen, IL-2 is produced within minutes and then acts in an autocrine fashion to promote the expansion of that T cell clone. IL-2 also stimulates the proliferation and lytic activity of NK cells. However, IL-2 plays an important role in negatively regulating or terminating an inappropriate T cell response as well. When a cell is repeatedly stimulated with antigen, such as the case with autoantigens, IL-2 exposure renders the cells sensitive to a process called activation induced cell death (AICD) and promotes their elimination. Mice that are deficient for IL-2 or IL-2 receptor subunits develop autoimmunity suggesting an important role for IL-2 in regulating inappropriate T cell responses.

### IFNγ

As mentioned, IFNγ plays an important role in activating macrophages during the innate immune response as the downstream target of bacterially stimulated IL-12. However, IFNγ also directly influences the function of antigen-specific lymphocytes during an immune response. For example, IFNγ, along with IL-12, directly promotes the differentiation of CD4+ T cells to become IFNγ-producing Th1 effector cells (Figure 1). As discussed earlier, this T cell subset is critical for the clearance of intracellular bacteria. IFNγ also acts directly on B lymphocytes to promote the production of the specific IgG subclass, IgG2a. This IgG subclass binds to Fcγ receptors on macrophages and promotes phagocytosis of microbes. IFNγ also has indirect effects on T cell function through the up-regulation of MHC molecules on antigen presenting cells, which ultimately increases the opportunity for antigen recognition by the T cell.

### IL-4

IL-4 is a cytokine that acts in many ways as an antagonist to the actions of IFNγ described previously. As opposed to the Th1-promoting properties of IFNγ, IL-4 directly promotes the differentiation of CD4+ T cells to become IL-4-producing Th2 cells and inhibits the ability of IFNγ to promote Th1 differentiation (Figure 1). As described, Th2 cells are critical for the clearance of

parasitic infections. IL-4 also promotes the production of the IgE immuno-globulin isotype, which is important for mast cell-mediated immune responses, and inhibits class switching to the IFN$\gamma$-stimulated IgG2a. IL-4 has also been shown to inhibit the ability of IFN$\gamma$ to activate macrophages but promotes MHC class II expression on antigen presenting cells.

### Regulatory Cytokines

Most of the features of cytokines described here thus far have involved stimulating the proliferation, differentiation, or microbicidal potential of a responding cell. Recently it has become apparent that certain cytokines have a more regulatory nature. Two of the cytokines that have garnered increased interest recently are TGF-$\beta$ and IL-10. TGF-$\beta$ generally inhibits the proliferation and activation of lymphocytes and macrophages. TGF-$\beta$ also stimulates the production of the immunoglobulin isotype IgA, which plays an important role in mucosal immunity. IL-10 has been described primarily as an inhibitor of macrophage function. Exposure of a macrophage to IL-10 results in decreased IL-12 and TNF production and decreased MHC expression. This decrease in macrophage function then in turn leads to decreased T lymphocyte responses as well. Mice that are deficient for IL-10 develop severe inflammatory bowel disease suggestive of uncontrolled macrophage activation. The expression of these regulatory cytokines have been ascribed to certain T cell subsets (T-regs) thought to be important for specifically suppressing immune responses to autoantigens and preventing the inappropriate activation of bystander lymphocytes.

### Cytokines and Immunopathology

As important as cytokines are in orchestrating an immune response, dysregulated cytokine production can have serious and deleterious results. For example, the tissue destruction associated with autoimmune diseases such as type 1 diabetes and rheumatoid arthritis is strongly associated with elevated expression of proinflammatory cytokines such as IFN$\gamma$ and TNF. Additionally, the airway hyper-responsiveness associated with allergic asthma has been causally linked with the Th2 cytokine IL-13. The notion of a relationship between dysregulated cytokine expression and disease has resulted in clinical applications. In particular, anti-TNF therapies have been effective in treating autoimmune disorders such as rheumatoid arthritis

## Cytokine Receptors and Signaling

Cytokines mediate their biological effect through the binding of specific receptors on target cell surfaces. These receptors then convert an external signal (binding of the cytokine to the receptor) to an intracellular biochemical signal, usually resulting in new gene transcription. Cytokine receptors are transmembrane proteins where binding of the cytokine occurs in the extracellular region and interaction with signaling proteins occurs in the cytoplasm.

### Cytokine Receptor Classes

Classification of cytokine receptors is based on structural homologies in the cytokine-binding regions. There are five families of cytokine receptors: type I cytokine receptors (IL-2 and IL-4), type II cytokine receptors (type I IFNs and IL-10), Ig superfamily receptors (IL-1), TNF receptors, and serpentine receptors (chemokines).

### Cytokine Receptor Expression

Like the highly regulated expression of cytokines described above, expression of the cytokine receptors themselves is also highly regulated and can influence the cellular response to the cytokine. For example, the IL-2 receptor is composed of three polypeptide chains; $\alpha$, $\beta$, and $\gamma$. In order for the T cell to maximally respond to IL-2 all three receptor chains must be expressed. When a T cell is specifically activated by antigen, the expression of the $\alpha$-chain is induced and the cell is capable of expanding to the IL-2 it produces. This provides a link between the antigen specific activation of the T cell and the ability of that cell to subsequently respond to IL-2 ensuring that antigen specific T cells are preferentially and appropriately expanded during an immune response.

### JAK/STAT Signaling

The best-defined signaling pathway utilized by type I and type II cytokine receptors involves the activation of Janus kinases (JAKs) and transcription factors called signal transducers and activators of transcription (STATs) (Figure 3). The JAK/STAT signaling pathway provides a direct link between cytokine binding at the cell surface to changes in gene expression at the level of new gene transcription. The general scheme consists of inactive JAK kinases bound specifically to the cytokine receptor cytoplasmic tail. Upon cytokine binding, the receptor chains are brought together and induce the activation of the JAKs through transphosphorylation. The JAKs also phosphorylate specific tyrosine residues on the cytoplasmic portion of the cytokine receptor, serving as a docking site for specific STAT proteins. The STAT proteins are also then phosphorylated, dimerize, and migrate to the nucleus. The dimerized STAT proteins are able to bind to specific DNA sequences in

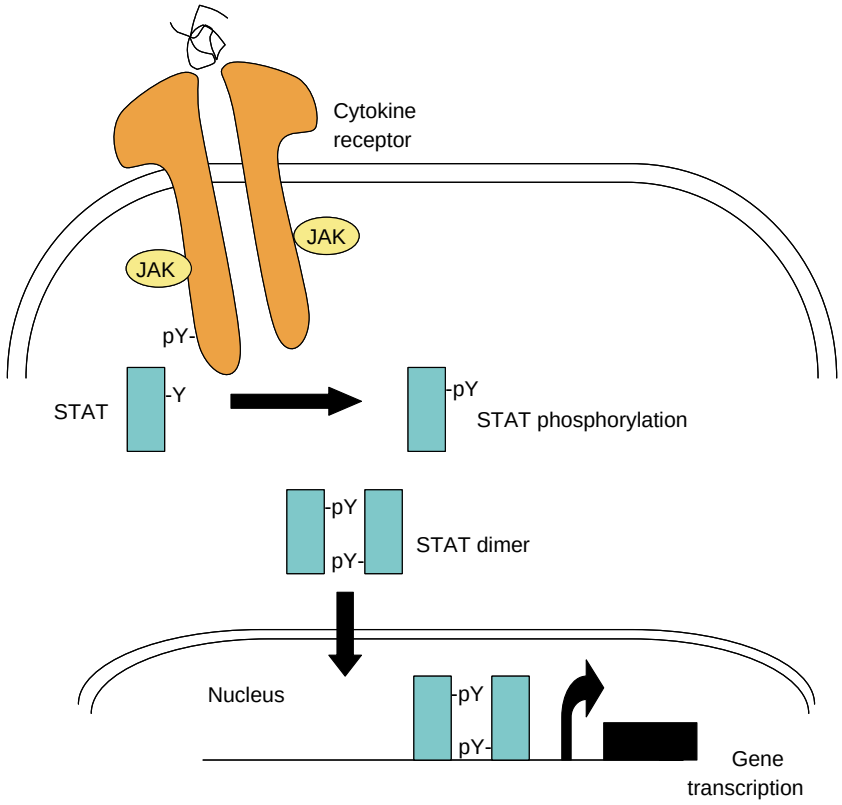



**FIGURE 3** JAK/STAT signaling pathway. The activation of the JAKs after cytokine stimulation results in the tyrosine phosphorylation of the cytoplasmic tail of the cytokine receptor and recruitment of the STAT protein. The STAT protein is also tyrosine phosphorylated, forms dimers and migrates to the nucleus to activate the transcription of cytokine responsive genes.

the promoters of cytokine inducible genes and activate the transcription of those genes directly. Different cytokine receptors bind to unique JAK and STAT combinations resulting in specific outcomes to cytokine exposure.

## Negative Regulation of Cytokine Signaling

Unopposed cytokine signaling can lead to a number of immune disorders so several regulatory mechanisms are in place to turn off JAK/STAT signaling after cytokine exposure. First, a family of cytoplasmic proteins called suppressor of cytokine signaling (SOCS) can directly interfere with JAK activity or with STAT binding to the cytokine receptor. The expression of the SOCS genes themselves is directly activated by the cytokine-induced STATs, consequently forming a classic negative feedback loop. SOCS1-deficient mice die within a few weeks of birth due to uncontrolled IFN$\gamma$ responses suggesting a critical role for SOCS proteins in regulating cytokine signaling. JAK and STAT activity can also be negatively regulated by protein tyrosine phosphatases, which return the JAK and STAT proteins to their unphosphorylated, inactive states. STATs are also negatively regulated by their interaction with members of the PIAS protein family. The PIAS proteins in general act to inhibit the ability of STAT proteins to stimulate gene transcription.

## See Also the Following Articles

Immunoglobulin (Fc) Receptors • Interferon Receptors • JAK-STAT Signaling Paradigm • Septins and Cytokinesis • T-Cell Antigen Receptor

## Glossary

**adaptive immunity** Immunity mediated by lymphocytes after exposure to foreign pathogen. Characterized by exquisite specificity for antigen and the formation of a memory immune response.

**innate immunity** The initial and rapid response to invading microbes. The cell types involved include macrophages, NK cells, and neutrophils. The mechanism for response exists before infection and does not result in immunological memory.

**JAK/STAT pathway** Intracellular signaling pathway induced by triggering of type I and type II cytokine receptors. Involves activation of specific kinases (JAKs), recruitment, and phosphorylation of transcription factors (STATs) and their subsequent

translocation as active dimers to the nucleus to activate gene transcription of cytokine responsive genes.

**macrophage** A phagocytic cell that plays important roles in both adaptive and innate immune responses. Activated macrophages engulf and kill invading microorganisms, secrete cytokines, and present antigen to T helper cells.

**T helper (Th) cell** CD4+ T cell subset that mediates cell-mediated responses in the adaptive immune system. Th cells are divided into two distinct subsets (Th1 and Th2) depending on the cytokines they secrete.

## Further Reading

Hill, N., and Sarvetnick, N. (2002). Cytokines: Promoters and dampeners of autoimmunity. *Curr. Opin. Immunol.* **14**, 791–797.

Murphy, K., and Reiner, S. (2002). Decision making in the immune system: The lineage decisions of helper T cells. *Nat. Rev. Immunol.* **2**, 933–944.

Renauld, J.-C. (2003). Class II cytokine receptors and their ligands: Key antiviral and inflammatory modulators. *Nat. Rev. Immunol.* **3**, 667–676.

Shuai, K., and Liu, B. (2003). Regulation of JAK–STAT signaling in the immune system. *Nat. Rev. Immunol.* **3**, 900–911.

## Biography

Andrea Wurster is an Instructor at the Harvard Medical School. She holds a Ph.D. from the University of California, San Diego. Her research has focused on the role of STAT proteins and cytokines in immune responses.

Michael Grusby is a Professor of Molecular Immunology at the Harvard School of Public Health. He received his Ph.D. from Northwestern University and serves on the editorial boards for the journals *Immunity* and *Journal of Biological Chemistry*. His research has focused on the role STAT proteins and cytokines in immune responses.

# Cytokinesis

**Masanori Mishima and Michael Glotzer**
*Research Institute for Molecular Pathology, Vienna, Austria*

**During cell division the chromosomes are equally segregated to the two poles of the mitotic spindle and the resulting two sets of chromosomes are sequestered into separate cells by a process known as cytokinesis. Cytokinesis must be controlled in both space and time so that the resulting daughter cells each contain a complete copy of the genetic material and other cellular organelles.**

## Making the Plans: Various Strategies for Coordinating Nuclear and Cytoplasmic Division

Diverse biological constraints dictate that cytokinesis is performed in different ways in various organisms. For example, the presence or absence of a cell wall greatly influences the nature of the cytokinetic process. In plant cells, which have cell walls, the daughter cells are separated by the cell plate, which is formed in the center of the cell by vesicle transport and fusion. In animal cells, the cleavage furrow is generated by constriction of a contractile ring containing actin and myosin II as key components.

A second feature which appears to differ among different cell types is the means by which the division plane is coordinated with nuclear division. For example, in plants and yeast, the division plane is established before mitosis and chromosome segregation is coordinated with this pre-existing spatial determinant. In contrast, in animal cells, the position of the division plane is established by the mitotic spindle in anaphase.

On the other hand, recent data indicate that different modes of cytokinesis rely on common molecular mechanisms. In yeast, cell-wall synthesis had been thought to be the major mechanism of cytokinesis. However, ingression of cell membrane by the constriction of actin ring is now known also to be important. Conversely, in animal cells, although the dominant factor in cell division is constriction of the contractile ring, vesicle trafficking has recently been demonstrated to contribute to this process. A comparison of the molecules required for cytokinesis in various organisms reveals that although some proteins, such as myosin and profilin, are generally required for cytokinesis in all species analyzed thus far, other proteins that are essential in one system are sometimes not even present in other systems (Table I). Thus, the different modes of cytokinesis in various systems may reflect varying degrees of reliance on common basic processes.

Identification and characterization of the molecular machines responsible for cytokinesis has advanced greatly because of the use of genetic analysis in model organisms including yeast, flies, and worms. It is anticipated that the use of RNA-mediated gene silencing (RNAi) will significantly enhance the understanding of cytokinesis in mammalian cells. The proteins involved in cytokinesis conserved among model organisms are summarized in Table I. The mechanism of cytokinesis in animal cells is the primary focus of this article (Figure 1).

## Making the Plans: Coordinating Nuclear and Cytoplasmic Division in Animal Cells

In animal cells, the mitotic spindle provides the spatial cue for division. Micromanipulation experiments indicate that microtubules are the critical factor within the spindle; the chromosomes are dispensible. Although it was long thought that microtubules provide a positive signal for cleavage furrow formation, recent evidence suggests that microtubules may generally inhibit cortical contractility and that a local minimum of microtubule density may direct cleavage furrow positioning. However, the underlying molecular mechanism has not been determined.

Since the critical factor required for contractile ring formation is RhoA, a simple, speculative model that could account for cleavage furrow formation is that high microtubule density inhibits RhoA activation and that sites where microtubule density reaches a local minimum induce activation of the small GTPase, RhoA. Like other small GTPases, the activity of RhoA is controlled by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Proteins Involved in Cytokinesis Conserved Among Model Organisms**

| Family | Domains | Mammal | Drosophila | *C. elegans* | *S. pombe* | *S. cerevisiae* | Dictyostelium |
|---|---|---|---|---|---|---|---|
| *Contractile ring* | | | | | | | |
| Myosin II heavy chain | MYSc, CC | Cytoplasmic myosin II | Zipper | NMY-2 | Myo2, Myo3/Myp2 | MYO1 | MHCA |
| Myosin II light chains | EFh | Essential LC, regulatory LC | Spaghetti squash | MLC-4 | Cdc4, Rlc1 | Mlc1, Mlc2 | Essential LC, regulatory LC |
| Formin | FH1, FH2, FH3 | mDia1, mDia2 | Diaphanous, Cappuccino | CYK-1 | Cdc12 | Bni1, Bnr1 | (For A, B, C) |
| Profilin | PROF | Profilin | Chickadee | PFN-1 | Cdc3 | Pfy1 | Profilin I, II |
| Rho-kinase | S_TKc, CC, PH | Rho-kinase/ROCK | (Drok) | LET-502 | ? | ? | ? |
| Pebble/ECT2 | BRCT, RhoGEF | Ect2 | Pebble | LET-21 | ? | ? | ? |
| Cofilin/ADF | ADF | Cofilin/ADF | Twinstar | (UNC-60A, B) | (Cof1/Adf1) | (Cof1) | (Cofilin, cofilin-2) |
| *Central spindle* | | | | | | | |
| PRC1/Ase1 | (a Map) | PRC1 | (CG11207, CG1655) | (Y34D9A.4) | (CAC21482) | Ase1 | ? |
| Centralspindlin kinesin | KISc, CC | MKLP-1/CHO1 | Pavarotti | ZEN-4 | ? | ? | ? |
| Centralspindlin RhoGAP | CC, C1, RhoGAP | MgcRacGAP/ HsCYK-4 | DRacGAP/acGAP | CYK-4 | ? | ? | ? |
| *Mitotic kinase/phosphatase* | | | | | | | |
| AuroraB | S_TKc | AuroraB/ AIM-1/AIRK2 | DmAurora B/IAL | AIR-2 | (Ark1/Aim1) | (Ipl1) | ? |
| INCENP | IN box | INCENP | DmlNCENP | ICP-1 | (Pic1) | (Sli15) | ? |
| Survivin | BIR | Survivin | Survivin | BIR-1 | (Bir1/Cut17) | (Bir1) | ? |
| Polo kinase | S_TKc, polo box | PLK1 | Polo | PLK-1 | Plo1 | Cdc5 | ? |
| Cdc14 | Protein phosphatase | hCdc14A, B | (CG7134) | CeCDC-14 | Flp1/Clp1 | Cdc14 | ? |
| *Others* | | | | | | | |
| Septin | GTPase | (Nedd5, H5, Diff 6, MSF, etc.) | (Peanut, Sep1, Sep2) | (UNC-59, UNC-61) | Spn1-6 | Cdc3, 10, 11, 12 | ? |
| IQGAP | CH, IQ, RasGAP | (IQGAP1, 2) | ? | (F09C3.1?) | Rng2 | Cyk1/Iqg1 | GAPA, DGAP1 |
| Cdc15 | FCH, SH3 | (PSTPIP) | ? | ? | Cdc15, Imp2 | Cyk2/Hof1 | ? |

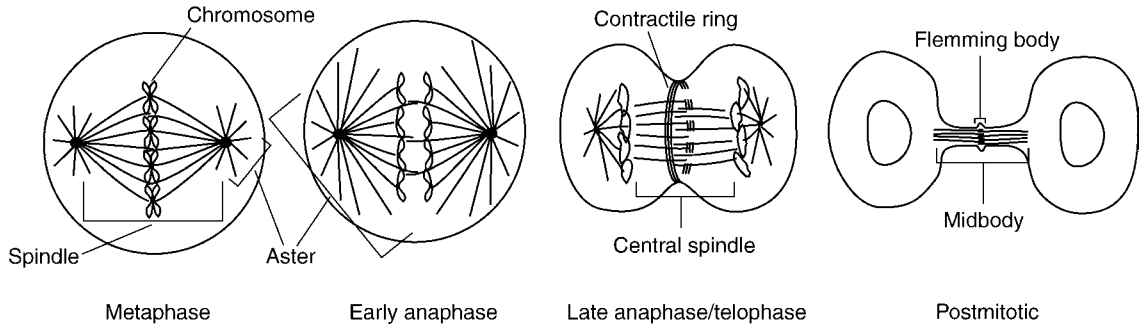



**FIGURE 1** The stages of cytokinesis and a guide to the cellular structures involved.

There is strong evidence that a specific GEF regulates cytokinesis. This protein, known as Pebble in flies or ECT2 in mammalian cells, is a multidomain protein whose loss of function parallels that of depletion of RhoA. However, it is not clear if the mitotic spindle generates local activation of RhoA by inducing high GEF activity at regions of low microtubule density. Alternatively RhoA could be activated globally, but other regulatory processes could inhibit the biological function of RhoA where microtubule density is high.

## Assembling the Parts 1: Building the Contractile Ring

A number of direct effectors of RhoA have been described and shown to be involved in cytokinesis, including Rho kinase and members of the formin family (Figure 2). Formin family members are auto-inhibitory proteins which are activated by binding of active RhoA. Formins are found concentrated at the contractile ring. They bind to an actin-binding protein, profilin, via a proline-rich sequence. Recently, formin proteins have been shown to have actin-polymerizing activity; this activity is further accelerated by profilin. Thus, formins could contribute to the formation of contractile ring by directing actin polymerization. There is functional evidence for a requirement for formin proteins in cytokinesis in mammals, flies, worms, budding yeast, and fission yeast; so far there is no evidence that formins are required for cytokinesis in plants or *Dictyostelium*.

Like formins, Rho kinase (ROCK) also localizes to the cleavage furrow. One substrate of this kinase is the activation site of the regulatory light chain of myosin II. ROCK also phosphorylates and inactivates the myosin-binding subunit of myosin phosphatase (MBS). Both pathways lead to the activation of myosin-II. In *C. elegans*, mutation of the ROCK orthologue, LET-502, causes defects in cytokinesis. However, these defects can be suppressed by mutation of MBS MEL-11. Thus, ROCK is an important player in cytokinesis, though perhaps it plays an accessory role rather than an essential one.

The contractile ring is a highly dynamic structure. Inhibition of actin polymerization during furrow ingression blocks propagation of the cleavage furrow. Conversely, depolymerization of actin filament in concert with the progression of the constriction is also important for cytokinesis. Indeed, actin depolymerizing factor is required for normal cytokinesis. Moreover, there are indications that inactivation of RhoA is required for completion of cytokinesis, since the CYK-4 RhoGAP is required for completion of cytokinesis.

## Assembling the Parts 2: Building the Central Spindle

The central spindle, or spindle midzone, is a barrel-like structure of microtubules formed between the segregating chromosomes during anaphase. The plus ends of nonkinetochore spindle microtubules are bundled to form antiparallel arrays in the spindle midzone. As the cleavage furrow ingresses, these microtubules become compacted. Finally, they form a structure called the midbody with completely constricted contractile ring overlaid by the still continuous cell membrane.

The importance of the central spindle on the promotion and completion (in some systems, initiation) of cytokinesis has been shown by micromanipulation and genetic analysis. For example, depletion or inactivation of *C. elegans* ZEN-4, a kinesin-like molecule that concentrates on the central spindle prevents formation of this structure in early embryos. Contractile ring formation and furrow ingression nevertheless occur. However, the furrow fails to complete and it ultimately regresses. In *Drosophila*, the central spindle seems to be required for early steps in cytokinesis since furrow ingression is not observed in embryos that have mutations in the pavarotti locus (Pav is the orthologue of *zen-4*).

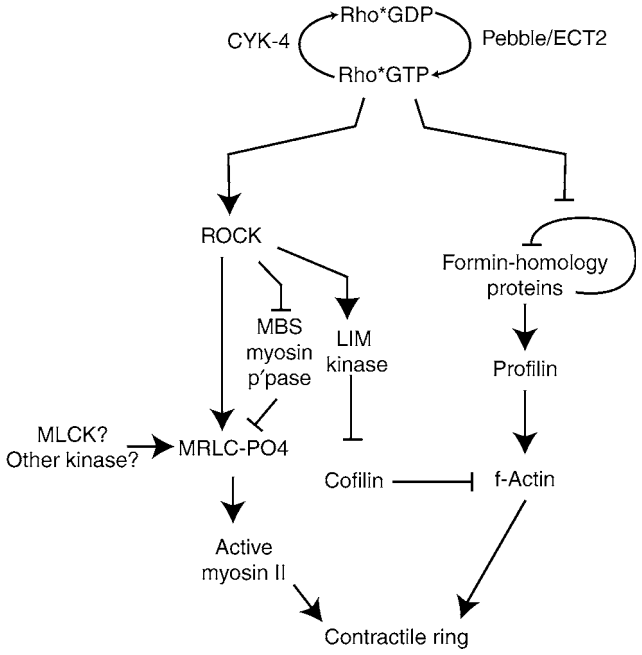



**FIGURE 2** The biochemical pathways through which RhoA regulates contractile ring formation.

A RhoGAP, CYK-4, colocalizes with ZEN-4 to the central spindle and midbody. Like ZEN-4, CYK-4 involved the formation of central spindle. CYK-4 forms a stoichiometric complex with ZEN-4 both *in vivo* and *in vitro*. A mutation in the CYK-4 binding domain of ZEN-4 can suppress the embryonic lethality caused by a mutation on the ZEN-4 binding domain of CYK-4, indicating the importance of complex formation for the *in vivo* function of these molecules. This complex is probably directly involved in assembling the central spindle since purified, recombinant CYK-4/ZEN-4 complex has microtubule bundling activity (Figure 3).

Human orthologues of CYK-4 and ZEN-4, HsCYK-4/MgcRacGAP and MKLP-1/CHO1, respectively, also form a similar protein complex. Depletion of HsCYK-4 by RNAi causes cytokinesis defects. Overexpression of MgcRacGAP mutant lacking MKLP-1 binding region or with mutation in the RhoGAP domain inhibits cytokinesis. Overexpression of MKLP-1 with a mutation in the motor domain causes an abnormal central spindle, loss of midbody material, and multinuclear cells. Thus, a protein complex, centralspindlin, containing orthologues of CYK-4 and ZEN-4 has an evolutionary conserved role in the formation and function of central spindle/midbody.

Another molecule, PRC1, is also important for the formation of central spindle. It too has microtubule bundling activity *in vitro*. Antibody injection or depletion by RNAi causes abnormal central spindle and inhibits cytokinesis. In metaphase, PRC1 is phosphorylated by cyclin-dependent kinase 1 (CDK1). This phosphorylation is thought to negatively regulate the protein because a mutant lacking the phosphorylation sites causes abnormal bundling of microtubules in the metaphase spindle. PRC1 contains a conserved central region. Other proteins that have this conserved domain are also implicated in organizing the anaphase spindle, e.g., budding yeast Ase1. In this context it is notable that the centralspindlin complex is not present in yeast. There is no evidence that the anaphase spindle participates in cytokinesis in budding or fission yeast.

Although the mechanism of formation of central spindle has been gradually revealed, the function of the central spindle in cytokinesis remains unclear. There are at least three possibilities.

1. It could be involved in the determination of cleavage site by modulating the distribution of microtubules in the mitotic apparatus during anaphase.
2. It could serve to localize factors that regulate the assembly and/or disassembly of the contractile ring.
3. Like the phragmoplast in plants, it could function to direct vesicle traffic along the ordered microtubule bundles.

These possibilities are not mutually exclusive, although the relative importance might vary among cell types.

## Pushing the Envelope: Membrane Fusion

Geometrical considerations require that during cytokinesis, if the total volume of the daughter cells is equivalent to that of the parental cell, the surface area of the cells must increase. Indeed, evidence has accumulated suggesting that vesicle traffic plays an important role in cytokinesis. In *Xenopus*, Zebrafish, and sea urchin embryos, insertion of new cell membrane into cleavage furrow by exocytosis has been observed.

The machinery responsible for this membrane insertion has begun to emerge. Specific syntaxins, rabs, and Golgi proteins have been implicated in cytokinesis and/or cellularization in *Drosophila*, *C. elegans*, and sea urchin embryos. Specific Golgi proteins, such as Lava Lamp, which interacts both with actin and microtubules, are required for cellularization. A lipid kinase, phosphatidylinositol 4-kinase, is required for cytokinesis in spermatocytes (the cell-type specificity may result from genetic redundancy in other cell types).

There are other interesting observations from the point of view of lipid membrane and cytokinesis. Phosphatidylethanolamine (PE) appears on the outer leaflet of cell membrane of cleavage furrow while it is usually enriched in the inner leaflet. A multivalent

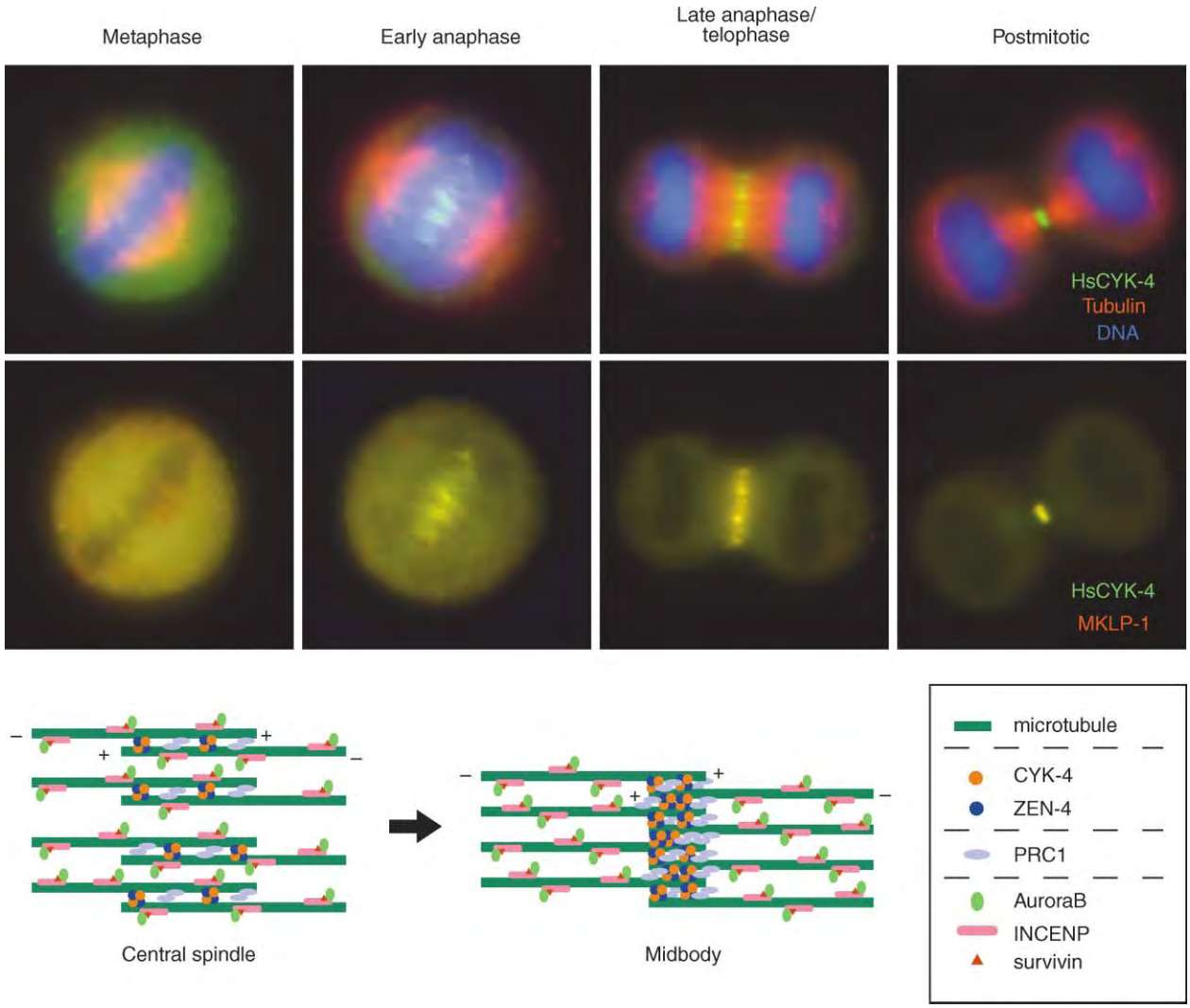



**FIGURE 3** The localization of the centralspindlin components MKLP1/HsCYK-4 during the process of cytokinesis. A schematic drawing depicting the assembly of the central spindle.

PE-binding peptide inhibits depolymerization of actin filament at midbody stage, and prevents completion of cytokinesis. Likewise, a sphingolipid, psychosine, causes defect of cytokinesis in some cells.

## Getting the Timing Right

In anaphase, activation of anaphase-promoting complex (APC) induces segregation of chromosomes and exit from mitosis by catalyzing the proteolytic destruction of securin and cyclins. Because cytokinesis occurs once per cell cycle and it is coupled to the exit from mitosis, there must be some connection between the cell-cycle engine and cytokinesis. That this is indeed the case is indicated by the fact that a nondegradable mutant of cyclin B prevents assembly of the central spindle and inhibits cytokinesis in mammalian cells, *Drosophila* and sea urchin embryos. This suggests that CDK1/cyclin B negatively regulates cytokinesis. This may be in part due to the regulation of microtubule dynamics. If the spindle is displaced to the vicinity of the cell cortex, furrow ingression can occur in the presence of high levels of CDK1/cyclin B.

CDK1/cyclin B may also regulate central spindle assembly via PRC1, since its function appears to be negatively regulated by CDK1. The RhoGEF, Ect2/Pebble, may also be negatively regulated by CDK1/cyclin B. In addition, cyclic activation of myosin-II in *Drosophila* embryos is indirectly regulated by CDK1 activity.

A conserved signaling cascade called the mitotic exit network (MEN) (budding yeast) or the septation initiation network (SIN) (fission yeast) has been

characterized in detail. At the end of the cascade, a protein phosphatase (Cdc14 in budding yeast and Flp1/Clp1 in fission yeast) is activated. In budding yeast, activation of Cdc14 leads to the activation of APC–Cdh1 through the dephosphorylation of Cdh1. In *S. pombe*, Flp1/Clp1 is involved in the regulation of the timing of septum formation and entry into M-phase. Although their roles are apparently different, antagonism of CDK1 activity could be a common function between Cdc14 and Flp1/Clp1. There are orthologues of Cdc14 in animal cells; however, the conservation of the MEN or SIN cascade is less clear. In mammalian cells, inhibition of Cdc14 causes several mitotic defects including abnormal separation of centrosomes and failure of the completion of cytokinesis. In nematodes, CeCDC-14 localizes to central spindle and its depletion by RNAi causes a similar phenotype to depletion of ZEN-4. Perhaps one conserved role of CeCDC-14 is to relieve the inhibition of cytokinesis by CDK1, though the critical targets may be different in different systems.

## Finishing the Job

Daughter cells typically enter $G_1$ phase connected to each other by the midbody. How they are finally separated (abscission) is not clear, although there is evidence that the midbody and vesicle traffic are involved. Interestingly, it has been observed that the abscission is delayed while the mother centriole, which is loosely connected to the daughter centriole, comes close to the midbody. It has been observed that the timing of abscission correlates with the moment at which the mother centriole moves away from the midbody.

## See Also the Following Articles

Mitosis • Rho GTPases and Actin Cytoskeleton Dynamics

## Glossary

**aster** Radial array of microtubules that surrounds each of the spindle poles during mitosis.

**central spindle** Antiparallel bundle of microtubules that forms in anaphase between the segregating chromosomes.

**contractile ring** Actomyosin-based structure responsible for constricting the cell cortex.

**Flemming body** The phase dense structure found in the center of the midbody at late times in cytokinesis.

**midbody** The thin intercellular bridge connecting the two daughter cells.

## Further Reading

Finger, F. P., and White, J. G. (2002). Fusion and fission: Membrane trafficking in animal cytokinesis. *Cell* **108**, 727–730.

Glotzer, M. (2001). Animal cell cytokinesis. *Annu. Rev. Cell Dev. Biol.* **17**, 351–386.

McCollum, D., and Gould, K. L. (2001). Timing is everything: Regulation of mitotic exit and cytokinesis by the MEN and SIN. *Trends Cell Biol.* **11**, 89–95.

Severson, A. F., and Bowerman, B. (2002). Cytokinesis: Closing in on the central spindle. *Dev. Cell* **2**, 4–6.

Straight, A. F., and Field, C. M. (2000). Microtubules, membranes and cytokinesis. *Curr. Biol.* **10**, R760–R770.

## Biography

Masanori Mishima is a postdoctoral Fellow at the Research Institute for Molecular Pathology (IMP) in Vienna, Austria. He received his D.Sc. from the University of Tokyo.

Michael Glotzer is a Research Group Leader at the IMP in Vienna, Austria. He received his Ph.D. from the University of California, San Francisco. The Glotzer group studies cytokinesis using *C. elegans* as a principal model system.

# Cytokinin

**Thomas Schmülling**
*Free University of Berlin, Berlin, Germany*

**Cytokinins are plant-specific chemical messengers (hormones) that play a central role in the regulation of the plant cell cycle and numerous developmental processes. Cytokinins were discovered by F. Skoog, C. Miller, and co-workers during the 1950s as factors that promote cell division (cytokinesis). The first cytokinin discovered was an adenine (aminopurine) derivative named kinetin (6-furfuryl-aminopurine), which was isolated as a DNA degradation product. The first common natural cytokinin identified was purified from immature maize kernels and named zeatin. Several other cytokinins with related structures are known today. Cytokinins are present in all plant tissues. They are abundant in the root tip, shoot apex, and immature seeds. Their endogenous concentration is in the low nM range. Typically, several types of cytokinins and their modified forms are present in a given tissue. Cytokinins can act over long distances or in the direct vicinity of the cytokinin producing cells (paracrine signaling). Cytokinins may act also on the cell that produced them (autocrine signaling). Cytokinins are also produced by cyanobacteria, some plant pathogenic bacteria (e.g., *Agrobacterium tumefaciens*, *Pseudomonas savastanoi*, *Rhodococcus fascians*) and the slime-mold *Dictyostelium discoideum*.**

## Cytokinin Structures

Naturally occuring cytokinins are adenine derivatives with a side chain at the $N^6$-position (Figure 1). The structure and conformation of the $N^6$-attached side chain can markedly influence the biological activity of the cytokinin. Depending on the structure of the $N^6$-substituent, cytokinins are classified as isoprenoid or aromatic cytokinins. The biological activities of both classes are qualitatively similar but they may differ quantitatively in different processes. Isoprenoid cytokinins are the most abundant class. They are either isopentenyl (iP)-type cytokinins, having an isopentenyl $N^6$-side chain, or zeatin-type cytokinins, having a hydroxylated isopentenyl $N^6$-side chain. The side chain of a zeatin-type cytokinin occurs in either *cis* or *trans* configuration, depending on which of the two methyl groups is hydroxylated. The *cis* form is usually much less active. Reduction of the double bond in the side chain leads to dihydrozeatin. Aromatic cytokinins have an aromatic benzyl group at $N^6$. They occur more rarely and much less is known about them. Because of their greater stability, aromatic cytokinins are often used in tissue culture, an example is benzyladenine. In addition, there are the structurally unrelated phenylurea-type cytokinins (e.g., diphenylurea, thidiazuron), a class of synthetic cytokinins. These cytokinins are highly active but do not occur naturally.

## Cytokinin Biosynthesis and Metabolism

The rate of *de novo* synthesis, metabolic interconversion, and breakdown are, together with transport processes, relevant to the regulation of cytokinin homeostasis in cells. Cytokinin metabolism includes mainly conversions among cytokinin bases, ribosides, ribotides, side-chain modification, conjugation and conjugate-hydrolyzing reactions, and cytokinin degradation.

### Biosynthesis

The initial and rate-limiting step of biosynthesis of isoprenoid-type cytokinins is the transfer of the isopentenyl moiety from dimethylallyl pyrophosphate (DMAPP) to AMP, ADP or ATP. The reaction is catalyzed by DMAPP::AMP/ADP/ATP isopentenyltransferases (IPT). ADP and ATP are the preferred substrates of most of the known plant IPT enzymes, while bacterial enzymes prefer AMP. The reaction leads to the formation of isopentenyl-AMP, -ADP and -ATP, which are the precursor molecules of biologically active cytokinins. The isopentenyl side chain is subsequently hydroxylated to form zeatin-type cytokinins (Figure 1). An alternative pathway, in which an already hydroxylated side chain is directly added to the $N^6$-position of the adenine moiety, may exist. IPT enzymes are encoded in *Arabidopsis* by a small gene family with seven members (*AtIPT1*, *AtIPT3–AtIPT8*). *AtIPT* genes are expressed in specific tissues of the root and shoot (e.g., vasculature), indicating that cytokinin synthesis

 © 2004, Elsevier Inc. All Rights Reserved. 

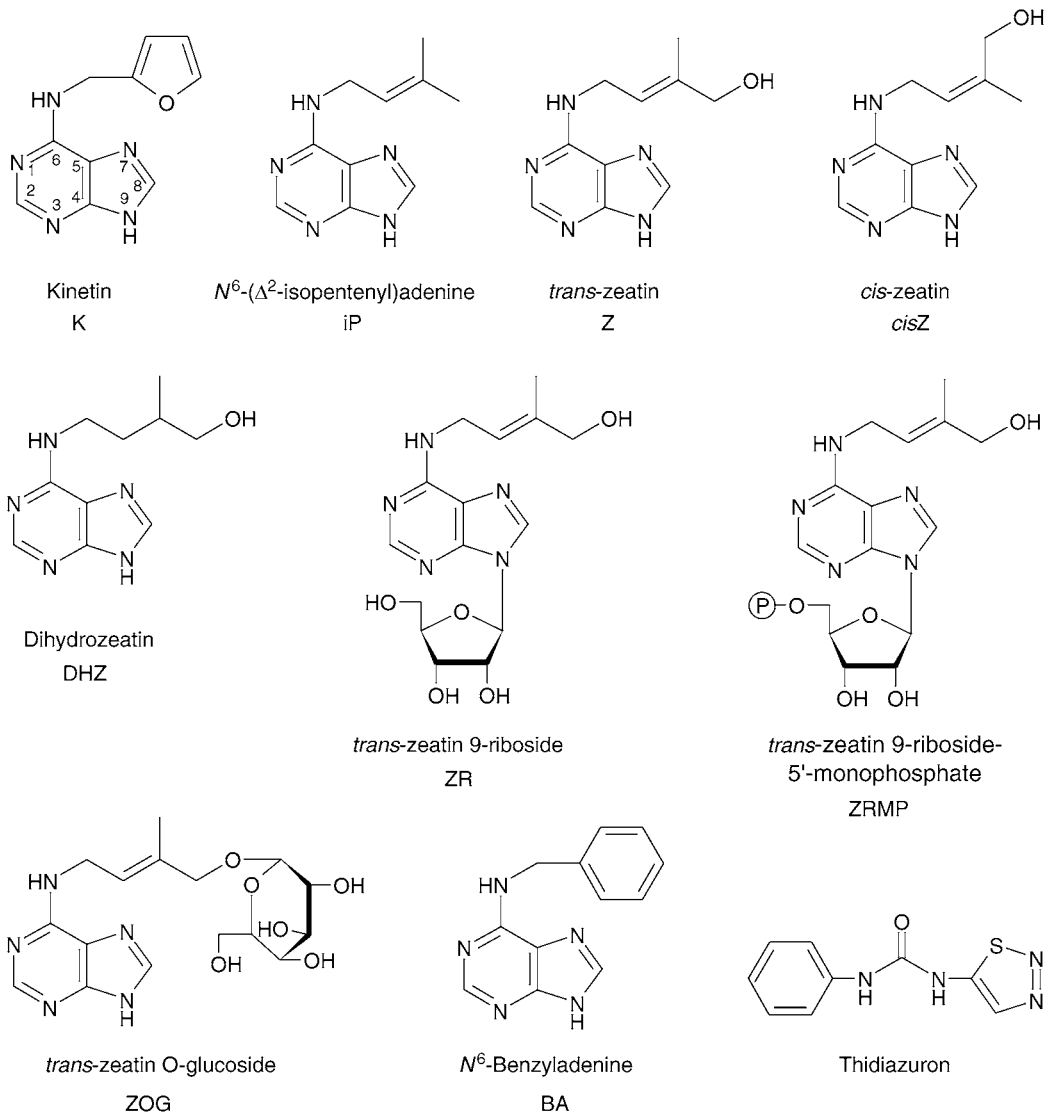



**FIGURE 1** Chemical structures of some naturally occurring and synthetic cytokinins. Common names and abbreviations are indicated below the structures. The numbering of purine ring atoms is shown for kinetin.

occurs in all major organs. Another possible source of cytokinins is tRNA, since tRNAs of most organisms contain isopentenylated adenine and other structural derivatives with cytokinin activity. However, it is generally assumed that tRNAs play only a minor role, if any, as a cytokinin source.

## Interconversion

A characteristic feature of cytokinin metabolism is the rapid metabolic interconversion of base, ribosides, and ribotides. The biologically most active form of cytokinins is the base. Attachment of ribose or ribose-5′-phosphate to the $N^9$ atom of the adenine ring leads to the formation of ribosides and ribotides, respectively, which do have lower activities (Figure 1). Interconversion of cytokinins is presumably an important mechanism to regulate the concentration of active compounds. Cytokinin ribosides are probably relevant as a transport form. The interconversions may be catalyzed by the same enzymes that metabolize adenine, adenosine, and AMP. The conversion between the *cis*- and *trans*-isomers of zeatin is catalyzed by the enzyme *cis–trans* zeatin isomerase. Zeatin is converted to dihydrozeatin by a NADPH-dependent zeatin reductase.

## Conjugation

Cytokinins can be stably or transiently inactivated by glycosylation of the purine ring or of the side chain. The purine ring can be glycosylated at the $N^3$-, $N^7$-, and $N^9$-positions. In addition, the $N^6$-side chain group can

form *O*-glycosyl conjugates if it bears a hydroxyl-group. Most often, glucose is the conjugated sugar molecule, more rarely xylose is attached. $N^7$- and $N^9$-conjugates are biologically inactive and extremely stable. Thus, they are irreversibly inactivated cytokinins. $N^3$- and *O*-conjugates are biologically inactive but can be readily hydrolyzed. They are believed to be transient storage forms of cytokinins. Glycosyl conjugation is considered to be important in the regulation of cytokinin activity levels, at least in some tissues and species. Several genes coding for cytokinin glycosyltransferases and glycosidases have been identified. Some conjugates of cytokinins and amino acids (alanine) have been described as well.

### Catabolism

Cytokinins are irreversibly degraded in a single enzymatic step by oxidative cleavage of the $N^6$-side chain. The reaction is catalyzed by cytokinin oxidases/dehydrogenases (CKX), which contain FAD as a cofactor. The reaction products are adenine and an aldehyde. The preferred substrates of CKX are isopentenyladenine, zeatin, and their corresponding ribosides. Ribotides, *O*-glucosides, dihydrozeatin, and aromatic cytokinins are not degraded by CKX. The *Arabidopsis* genome contains seven *AtCKX* genes, which are preferentially expressed in zones of active cell division and growth. The corresponding enzymes are located in the endoplasmatic reticulum, in the apoplast, and in the vacuole.

## Cytokinin Transport

Cytokinins are transported from roots to shoots in the xylem, and in the opposite direction in the phloem. Transported cytokinins may have a role in coordinating root and shoot development, for example, by carrying information about nutrient availability. Multiple cellular importers and exporters are required to allow efficient mobilization and targeted translocation of cytokinins, but very little is known about cytokinin transporters. Transport studies indicate that a common $H^+$-coupled high-affinity purine transport system transports cytokinins.

## Cytokinin Signaling

The mechanism of cytokinin signaling is just beginning to emerge. The cytokinin signal is perceived and transduced by a multistep phosphorelay system through a complex form of the two-component system (TCS) pathway. The TCS is common among prokaryotes and lower eukaryotes, among the higher eukaryotes it is unique to plants. In this signaling system, a membrane-located receptor kinase with an extracellular ligand-recognition domain (sensor) dimerizes upon binding a ligand and autophosphorylates a histidine within its cytoplasmic transmitter domain. The phosphoryl group is first transferred to an aspartate residue within the receiver domain at the C terminus of the receptor and from there to a His-containing phosphotransmitter (Hpt), which ultimately phosphorylates and thus activates a response regulator (RR) at a central Asp residue (see Figure 2).

### Signal Perception

Cytokinin receptors are histidine kinases consisting of an extracellular sensing domain, a cytoplasmic histidine kinase transmitter and receiver domains. Three cytokinin receptors (CRE1/WOL/AHK4, AHK2, AHK3) have been identified in *Arabidopsis*. They all share a ~270 amino acid long extracellular cyclases/histidine kinases associated sensing extracellular (CHASE) domain, which presumably recognizes cytokinin. This domain might have been acquired by plants through lateral gene transfer from cyanobacteria. Loss-of-function mutants of CRE1/WOL/AHK4 lack the phloem in their primary roots, indicating a role for cytokinins in embryo development.

### Signal Transduction

Current knowledge suggests that downstream signaling components of the cytokinin signal-transduction pathway in *Arabidopsis* consist of five Hpt and 22 response regulators of the A- or B-type. Hpts transmit the signal from the receptor, which is presumably localized in the plasma membrane, to B-type RRs, which are in the nucleus. B-type RRs consist of an N-terminal receiver domain and a C-terminal output domain, containing a DNA recognition motif called GARP, which is distantly related to the Myb repeat. The DNA motif optimal for binding is 5′-(A/G)GAT(T/C)-3′. Activated B-type ARRs transcribe primary response genes of cytokinins. Some of the known primary response genes, which are rapidly and specifically up-regulated by cytokinins, code for type A response regulators. Type A RRs resemble type B RRs but lack the C-terminal DNA binding and activation domain. Type A RRs fulfill at least two different functions. On the one hand, they exert a negative feedback regulation of the cytokinin signaling pathway through protein–protein interaction. On the other hand, they mediate the cytokinin-dependent modulation of other pathways, e.g., light signaling. A-Type response regulators can be

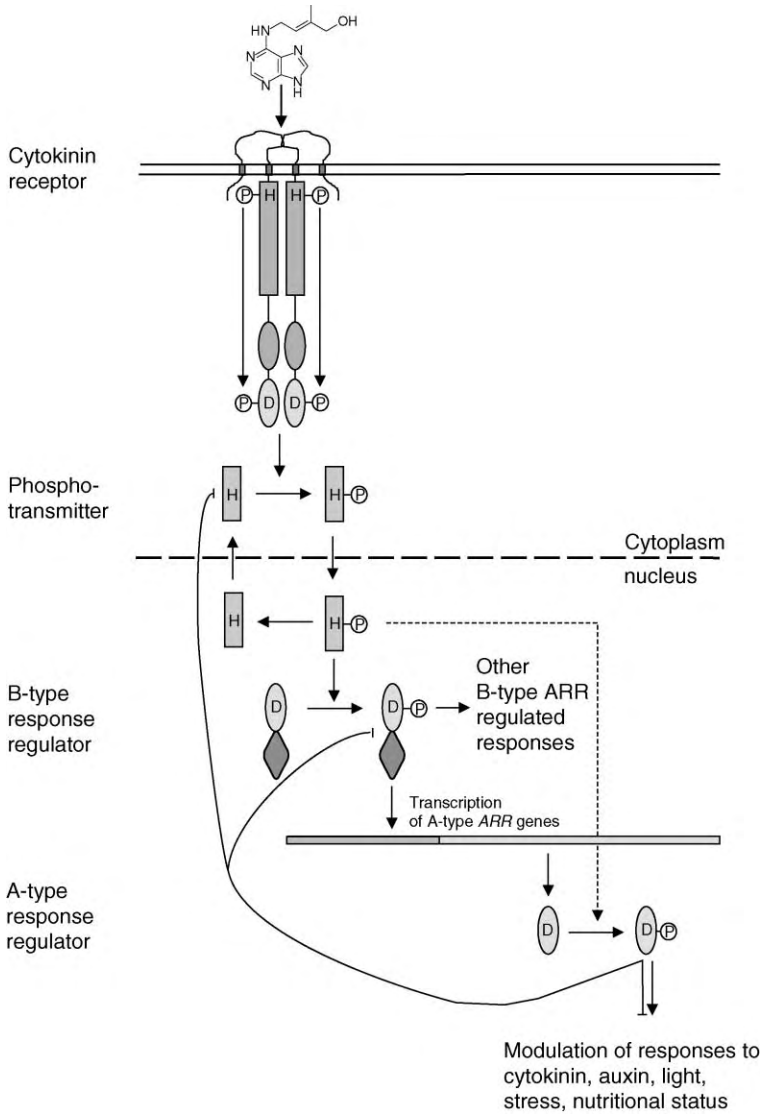



**FIGURE 2** A model for cytokinin signal transduction via a His-to-Asp phosphorelay. The structure of CRE1/WOL/AHK4 is shown as an example. Ligand binding induces receptor dimerization and autophosphorylation. Transfer of the phosphoryl group by activated receptors activates histidine phosphotransmitter proteins (Hpts), which transport the signal from the cytoplasm to B-type RRs in the nucleus. B-Type response regulators transcribe target genes, among them A-type *RR* genes. A-Type response regulators may down-regulate the primary cytokinin signal response via a negative feedback loop, modulate downstream activities of cytokinins in a positive or negative fashion, or modulate other signaling pathways through protein–protein interaction. A more complex regulation than shown in the model may exist. D, aspartate residue; H, histidine residue; P, phosphoryl group.

positive or negative regulators, depending on the individual response regulator and the output reaction analyzed.

## Cytokinin Functions

### Cell Cycle

Cytokinins are required for cell division during embryogenesis in the shoot apical meristem, young leaves, the cambium, and cultured plant cells. In contrast, they have a negative regulatory role in the root meristem. Cytokinin controls the exit of dividing cells from this meristem. Changes in cytokinin levels occur during the cell cycle of cultured cells, the level being highest during the late S and before the M phase. Cytokinins have been functionally linked to all stages of the cell cycle but their mechanism of action has only been partially elucidated. Cytokinin up-regulates expression of the D-type cyclin gene *CycD3*, which is important in regulating the G1/S-transition of the cell cycle. Cytokinin increases the number of replication origins during S-phase and it may also play a role in regulating G2/M transition.

## Plant Development and Growth

Cytokinin participates in regulating numerous aspects of plant development throughout the life cycle. These include seed germination, cotyledon expansion, chloroplast differentiation, de-etiolation, differentiation of vascular tissue, apical dominance (shoot branching), root elongation and branching, nutritional signaling, regulation of sink strength, the transition from the vegetative to the reproductive growth phase, flower and fruit development, leaf senescence, and plant–pathogen interactions. A role for the hormone in vascular morphogenesis during embryonic development is firmly established. During post-embryonic development cytokinins are required to maintain meristem activity and leaf development in the plant shoot. Local exogenous cytokinin application to the shoot leads to premature growth of lateral buds, retarded leaf senescence, partial photomorphogenesis in the dark, increased sink strength and an altered vasculature. In contrast to their stimulatory activities in the shoot, cytokinins have a negative regulatory role in the control of root elongation and branching. Additionally, cytokinin regulates important physiological parameters that determine biomass formation and distribution via central genes of primary metabolite pathways, including invertases, hexose transporters, and key genes of phosphate and nitrogen metabolism and signaling (e.g., nitrate reductase). Changes in cytokinin levels are generally positively correlated with levels of mineral nutrients, especially nitrogenous nutrients. Cytokinin levels are decreased by water stress. *In vitro*, the ratio of cytokinin to auxin determines the differentiation of cultured plant tissues to either shoots or roots. A high cytokinin to auxin ratio promotes shoot formation, a low ratio promotes root formation. Owing to their stimulatory effect on plant regeneration, cytokinins are widely used in plant tissue culture.

## Pathogenicity

Cytokinins are produced by several plant pathogenic bacteria and play a role in pathogenicity. One such pathogen is *Agrobacterium tumefaciens*, the causative agent of the crown gall disease. During the infection process, *A. tumefaciens* transfers a small stretch of DNA, the T-DNA, to the host plant, where it becomes integrated in the nuclear genome. The T-DNA harbors an *IPT* gene, which is expressed in the host cell and causes cytokinin overproduction. This leads, together with an enhanced auxin content, to tumorous cell proliferation. Other cytokinin-synthesizing pathogens are *Pseudomonas syringae*, which induces gall formation and *Rhodococcus fascians*, which causes fasciation and a growth abnormality called witch's broom disease. The root-nodule forming and nitrogen-fixing plant symbiont *Rhizobium spec.* is also known to produce cytokinin.

## Biotechnology

Practical use of cytokinin in agriculture is currently limited. Modulation of the endogenous cytokinin content of plants or interfering with cytokinin signaling has a high potential for biotechnological applications in agriculture. Plants with increased cytokinin content are more branched and senesce later. Moreover, cytokinins alter sink–source relations, a promising approach to improve yield attributes. Plants with reduced cytokinin content develop a larger root system. An improved root system means improved acquisition of minerals and water, factors which are often limiting for plant growth.

## See Also the Following Articles

Cytokines • Cytokinesis • Septins and Cytokinesis

## Glossary

**cell cycle** Sequence of events between mitotic divisions, divided into G1, (G standing for gap), S (synthesis phase), G2 and M (mitosis).

**cytokinin conjugate** Compound formed by the union of a cytokinin and a sugar moiety.

**meristem** Growing tip of roots and shoots.

**senescence** Programmed aging leading to organ or plant death.

**two-component system** Signal transduction system of bacteria, lower eukaryotes and plants; involves autophosphorylation of a histidine kinase that transmits the signal via phosphorelay to response regulator proteins.

## Further Reading

Davies, P. J. (1995). *Plant Hormones. Physiology, Biochemistry and Molecular Biology*. Kluwer Academic, Dordrecht.

Heyl, A., and Schmülling, T. (2003). Cytokinin signal perception and transduction. *Curr. Opin. Plant Biol.* **6**, 480–488.

Hoykaas, P. J. J., Hall, M. A., and Libbenga, K. R. (1999). *Biochemistry and Molecular Biology of Plant Hormones*. Elsevier, Amsterdam.

Kakimoto, T. (2003). Biosynthesis of cytokinins. *J. Plant. Res.* **116**, 233–239.

Kakimoto, T. (2003). Perception and signal transduction of cytokinins. *Ann. Rev. Plant Biol.* **54**, 605–627.

Kieber, J. J. (2002). Cytokinins. In *The Arabidopsis Book* (C. Somerville, E. Meyerowitz and M. D. Rockville, eds.) American Society of Plant Biologists, http://www.aspb.org/publications/arabidopsis.

Mok, D. W. S., and Mok, M. C. (1994). *Cytokinins. Chemistry, Activity, and Function*. CRC Press, Boca Raton, FL.

Mok, D. W. S., and Mok, M. C. (2001). Cytokinin metabolism and action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 89–118.

Schmülling, T., Werner, T., Riefler, M., Krupková, E., and Bartrina y Manns, I. (2003). Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. *J. Plant Res.* **116**, 241–252.

Taiz, L., and Zeiger, E. (2002). *Plant Physiology*. Sinauer, Sunderland, MA.

## Biography

Thomas Schmülling is a Professor of Genetics and Chair of Developmental Biology of Plants at the Free University of Berlin. His principal research field is the biology of cytokinins. He studied biology at the University of Cologne, accomplished his doctoral thesis at the Max Planck Institute for Plant Breeding and has been an Assistant Professor at the University of Tübingen. He has made contributions to the understanding of *Agrobacterium rhizogenes* T-DNA genes and cytokinin metabolism and signaling. His laboratory was the first to generate cytokinin-deficient plants by means of genetic engineering.

# Desmosomes and Hemidesmosomes

**Rachel L. Dusek, Jonathan C. R. Jones and Kathleen J. Green**
*Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA*

**Desmosomes and hemidesmosomes are cell junctions important for maintaining adherence within epithelial tissues, a function reflected in the Greek root *desmo*, meaning "bound." Desmosomes facilitate adhesion between adjacent epithelial cells, whereas hemidesmosomes (named for their ultrastructural resemblance to half a desmosome) mediate adhesion between basal cells of epithelial tissues and the substratum. These junctions are functionally alike in their ability to couple the intermediate filament (IF) cytoskeleton to sites of cell–cell or cell–substratum contact at the plasma membrane. However, these organelles differ dramatically in their molecular composition and specialized functions.**

## The Desmosome

Desmosomes are prominent in tissues that are subjected to mechanical stress. Found most abundantly in epithelial tissues, desmosomes are also present in the myocardium, brain meninges, and follicular dendritic cells of the lymph nodes. These specialized anchoring junctions function to mediate intercellular adhesion and maintain tissue integrity by connecting sites of cell–cell contact to the highly tensile intermediate filament (IF) cytoskeleton.

### Desmosome Ultrastructure

Ultrastructurally, the desmosome appears as a symmetrical, highly organized, electron-dense structure that connects neighboring cells (Figure 1A). The plasma membranes of adjacent cells are separated by an ~30-nm space containing an electron-dense midline, composed of the extracellular portion of the transmembrane desmosomal components. Just internal to the plasma membrane is an electron-dense outer plaque containing the intracellular desmosomal components. Interior to this region is a less-dense, IF-rich fibrillar inner plaque.

### Structure and Molecular Components of the Desmosome

The basic blueprint of a desmosome comprises molecular components that fall into three main gene families: desmosomal cadherins, armadillo family members, and plakins. Several studies suggest that these molecules are arranged linearly in this order from the cell surface inward and function together to indirectly tether the IF cytoskeleton to the cell membrane. Lateral interactions between these proteins occur as well and enhance the strength of the junction. Nonadhesive functions have also been proposed for several of these molecules. The best characterized desmosomal components (Figure 1B) are discussed in detail here. However, minor components, including pinin and desmocalmin/keratocalmin, have also been described.

#### Desmosomal Cadherins

Two types of glycosylated, type I transmembrane adhesive cadherin proteins are found in the desmosome, desmogleins (Dsgs), and desmocollins (Dscs). Different genes encode four isoforms of the Dsgs (1–4) and three isoforms of the Dscs (1–3), which are expressed differentially in various cell types and in a differentiation-specific manner in complex stratified epithelia. The Dsc isoforms are further subdivided into two types, a longer "a" form and a shorter "b" form. Like classical cadherins, both Dsgs and Dscs have a highly conserved calcium-binding extracellular domain, membrane-spanning region, and catenin-binding intracellular cadherin segment (ICS) (not present in Dsc b), whereas Dsgs contain additional unique cytoplasmic subdomains of unknown function. Dsgs and Dscs are thought to function primarily in mediating homo- and heterophilic calcium-dependent adhesion across the membranes of adjacent cells. Severe blistering of the skin or lesions on the hands and feet are consequences of genetic, autoimmune, and bacterial diseases that compromise the adhesive function of these molecules (Table I).

#### Armadillo Family Members

Proteins with homology to the *Drosophila* segment polarity protein armadillo are important structural components of the desmosome, linking the desmosomal cadherins at the plasma membrane with intracellular cytoskeleton components. These proteins contain

 © 2004, Elsevier Inc. All Rights Reserved. 

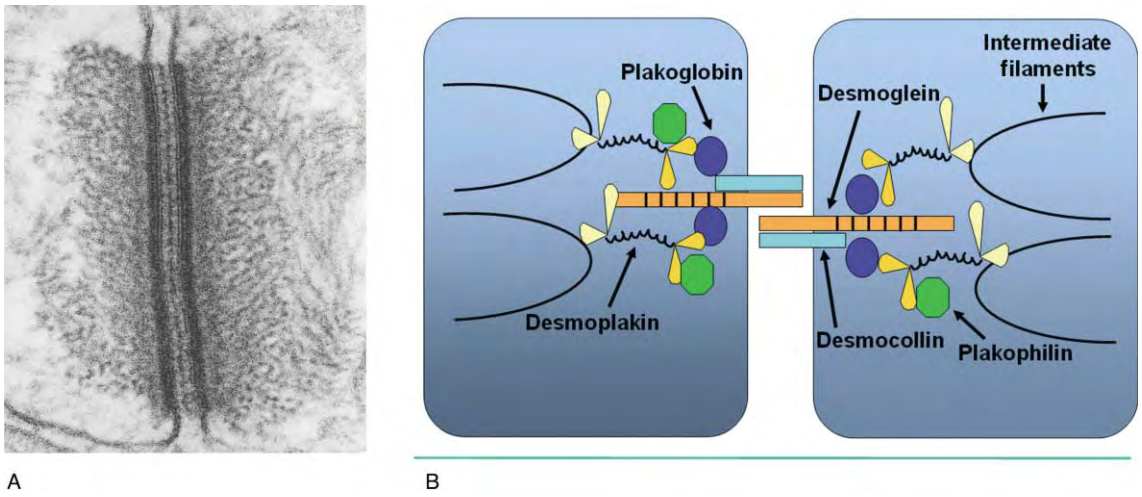



**FIGURE 1** Molecular composition and structure of the desmosome. (A) Desmosome ultrastructure is shown in this electron micrograph of bovine tongue epidermis. Reprinted from *Biophysical Chemistry*, Vol. 50, A. P. Kowalczyk, A.P., *et al*., Structure and function of desmosomal transmembrane core and plaque molecules, pp. 97–112, copyright 1994 with permission from Elsevier Science. (B) Desmosomal proteins, their interactions, and their approximate position within the organelle are depicted in this schematic diagram of the desmosome.

a 42-amino-acid repeated motif called the arm repeat, which is thought to mediate protein–protein interactions. Plakoglobin (Pg) is one armadillo protein whose presence in both adherens junctions and desmosomes is selectively regulated. In the desmosome, it binds directly to the cytoplasmic tail of the desmosomal cadherins and to the obligate desmosomal protein, desmoplakin (DP). Pg's adhesive importance is reflected by one genetic disease in which the disruption of its function results in cardiomyopathy, keratoderma, and wooly hair (Table I). In addition to its adhesive role, Pg is also thought to function in a variety of signal transduction pathways.

The plakophilins (PKP1–3) are another group of armadillo family members that contribute to desmosomal adhesion. These proteins have a specific tissue distribution and are differentially expressed in complex stratified epithelia. PKPs are found both in the desmosome and in the nucleus. In the desmosome, PKPs interact cytoplasmically with the desmosomal cadherins, as well as with DP and IFs. The role of PKPs in desmosome assembly and IF attachment is not entirely clear; however, PKP1 is known to enhance the recruitment of DP to the plasma membrane and to facilitate lateral clustering of plaque components. Genetic mutations in both PKP1 alleles negate the protein's function and result in excessive skin fragility (Table I).

### Plakins

Although desmosomes consist of various components in different tissues, DP, the most abundant protein in the desmosomal plaque, is an obligate desmosomal constituent. DP is the primary plakin family member found in the desmosome, although other less-abundant plakins include envoplakin, periplakin, and plectin. Structurally, the DP molecule consists of two globular ends connected by a coiled-coil rod whose size is modulated by alternative splicing of the transcript to produce two variants (I and II). The rod domain mediates DP self-dimerization. DP also directly associates with Pg and PKPs through its amino (N) terminus and with IFs through its carboxyl (C) terminus. In addition, direct association of DP with desmosomal cadherin tails has been noted. DP is required for desmosome assembly, linking the IF cytoskeleton to the plasma membrane and anchoring IFs in the plaque. The importance of DP in maintaining epithelial tissue integrity is emphasized by severe keratodermas, sometimes associated with heart defects, affecting patients with genetic diseases in which DP function is compromised by haploinsufficiency, protein truncation, or point mutations (Table I).

## Desmosome Assembly and Maintenance

The regulation of the assembly and disassembly of cell–cell junctions is important for normal tissue homeostasis and during junction-remodeling processes such as development or wound healing. One crucial factor controlling desmosome assembly appears to be the prerequisite formation of adherens junctions, perhaps to bring adjacent membranes close together and permit the interaction and clustering of desmosomal molecules. In cultured cells, desmosome assembly occurs in

**TABLE I**

**Human Diseases Associated with Desmosomal Components**

| Mutated gene/target antigen | Phenotype |
|---|---|
| **Genetic diseases** | |
| Plakoglobin | *Naxos disease*: Autosomal recessive arrythmogenic right ventricular cardiomyopathy (ARVC) combined with striate palmoplantar keratoderma (SPPK) and woolly hair |
| Plakophilin 1 | Autosomal recessive ectodermal dysplasia with a skin fragility syndrome, hair loss, reduced sweating, and nail dystrophy |
| Desmoglein 1 | *Striate palmoplantar keratoderma (SPPK)*: Lesions of the palms and soles exacerbated by mechanical trauma |
| Desmoglein 4 | *Autosomal recessive hypotrichosis* (LAH): Loss of hair on scalp, chest, arms, legs, and face due to hair follicle abnormalities |
| Desmoplakin (haploinsufficiency) | *Striate palmoplantar keratoderma (SPPK)*: Lesions of the palms and soles exacerbated by mechanical trauma |
| Desmoplakin (N-terminal missense mutation) | Autosomal dominant arrythmogenic right ventricular cardiomyopathy (ARVD/C) |
| Desmoplakin (C-terminal missense mutation) | Autosomal dominant arrhythmogenic right ventricular cardiomyopathy (ARVD/C); wooly hair and lesions on the palms of hands and soles of feet |
| Desmoplakin (C-terminal truncation) | Autosomal recessive left ventricular cardiomyopathy with SPPK and woolly hair |
| Desmoplakin (compound heterozygosities) | *Palmoplantar keratoderma*: One missense mutation in the N-terminus and one nonsense mutation resulting in a C-terminal deletion; alopecia, hyperkeratosis, acantholysis, and keratin retraction |
| **Autoimmune diseases** | |
| Desmoglein 3 | *Pemphigus vulgaris*: Blistering of the oral cavity caused by circulating autoantibodies directed against desmoglein 3; presence of antibodies against both desmoglein 3 and 1 cause the mucocutaneous form with blisters also in the deep epidermis |
| Desmoglein 1 | *Pemphigus foliaceus*: Blistering of the superficial epidermis caused by circulating autoantibodies directed against desmoglein 1 |
| Desmocollin 1, Desmoglein 1, Desmoglein 3 | *IgA pemphigus*: Intraepidermal blistering with different forms characterized by circulating autoantibodies against desmocollin 1, desmoglein 1, or desmoglein 3 |
| Plakin family members and desmogleins | *Paraneoplastic pemphigus*: Severe blistering of the skin and mucous membranes caused by circulating autoantibodies against plakin family proteins, desmoglein 1 and desmoglein 3; occurs mainly in patients suffering from malignant lymphomas and thymomas |

response to cell–cell contact in a calcium-dependent manner. In low-calcium conditions, newly synthesized desmosomal components are unstable. During assembly, desmosomal cadherins and Pg are delivered together in vesicles to the plasma membrane. Subsequently, DP and IFs are added to the assembling junction. Upon association with the IF cytoskeleton, desmosomal components become stabilized, insoluble, and lose their calcium dependence. The assembly and maintenance of desmosomes is regulated in part by post-translational modification of protein components. Specifically, serine/threonine and tyrosine phosphorylation of several desmosomal components regulates desmosomal assembly and maintenance by modulating the ability of desmosomal proteins to interact with one another.

## Desmosome Function

Far from static spot welds, desmosomes are malleable and dynamic structures that respond to extra- and intracellular signals to adapt their functions in cell–cell adhesion, morphogenesis, and signal transduction.

### Cell–Cell Adhesive Function

Although desmosomal cadherins were long assumed to function in calcium-dependent cell–cell adhesion, until recently there was a lack of direct evidence demonstrating that ectopically expressed desmosomal proteins promote adhesion in normally nonadherent cells. Evidence now suggests that both Dscs and Dsgs, along with the associated protein Pg, are required for adhesion and that they may interact heterophilically. The existence of autoimmune, genetic, and bacterial diseases that affect the function of desmosomal cadherins and result in compromised epidermal integrity (Table I) support the idea that desmosomes function in intercellular adhesion. Intercellular adhesion may also be disrupted by human diseases that perturb the structure of the desmosomal plaque (Table I). Cell culture models have demonstrated that IF attachment to the desmosome core is a critical aspect in the regulation of desmosome-mediated intercellular adhesion. This is especially important in tissues such as the heart, in which intercellular adhesive defects can have grave consequences.

### Morphogenetic Function

Desmosome-mediated intercellular adhesion may be intimately involved in many morphogenetic events. For example, because severe developmental abnormalities lead to embryonic lethality in DP-null mice, desmosomes are thought to play a critical role in maintaining tissue integrity during normal embryogenesis and development. In addition, the importance of desmosomal adhesion in the normal patterning of epithelial cells is demonstrated by the fact that peptide inhibition of desmosomal adhesion can disrupt mammary epithelial cell morphogenesis and positioning.

### Signaling Function

Desmosomes may also have a signaling function. A role in outside-in signaling is evidenced by the fact that the ligation of the extracellular domain of Dsg3 by pemphigus vulgaris (PV) antibodies can modulate the intracellular calcium concentration and phospholipid metabolism in keratinocytes. In cultured cells, such ligation results in serine phosphorylation of Dsg3 and its reported dissociation from Pg. Pg-null keratinocytes were used to demonstrate that PV antibody ligation of Dsg3 leads to a Pg-dependent keratin retraction, which may contribute to the epidermal-blistering characteristic of this disease. Intracellular signals can also be propagated by desmosomes. Pg, whether in a desmosomal or nondesmosomal pool, is thought to be the best candidate protein for this process. Although Pg can be found in different junction types, the metabolic stability and cellular localization of Pg are tightly regulated, allowing it to function in cell–cell adhesion, transcriptional activation, proliferation, and programmed cell death.

## The Hemidesmosome

Hemidesmosomes are specialized junctions that connect basal epithelial cells to the basement membrane and underlying extracellular matrix. Hemidesmosomes promote the integrity and strength of epithelial tissues by indirectly connecting the intracellular keratin IF cytoskeleton to extracellular matrix proteins.

### Hemidesmosome Ultrastructure

The ultrastructure of the hemidesmosome (Figure 2A) includes an electron-dense cytoplasmic triangular plaque, which makes up an inner IF-rich region closest to the cell cytoplasm and a perimembrane plaque containing the cytoplasmic tails of the transmembrane hemidesmosome components. Just adjacent and external to the basal cell membrane is a sub-basal dense plate and thin extracellular anchoring filaments that extend from the plate into the specialized extracellular matrix of the basement membrane.

### Structure and Molecular Components of the Hemidesmosome

The major hemidesmosomal components together connect the basal epithelial cell cytoskeleton to the extracellular matrix (Figure 2B). Hemidesmosomal proteins are arranged such that IFs are anchored to the plaque at multiple points, reenforcing the connection to the substratum.

#### $\alpha 6\beta 4$ Integrin

The $\alpha 6\beta 4$ integrin is the principal factor connecting IFs to the extracellular matrix and, as such, is essential for hemidesmosome formation and the maintenance of epithelial cell attachment to the basement membrane. The $\alpha 6$ integrin can dimerize with $\beta 1$ integrin, but prefers to interact noncovalently with $\beta 4$ integrin. The $\beta 4$ integrin has an unusually long cytoplasmic tail, important for the specialized functions of this molecule. The $\alpha 6\beta 4$ integrin pair is expressed predominantly in epithelial tissues, Schwann cells, and endothelial cells. In complex stratified epithelia, $\alpha 6\beta 4$ integrin is oriented in a polarized fashion on the basal surface of the basal cell layer. The association of the $\alpha 6$ and $\beta 4$ subunits generates a functional transmembrane receptor whose extracellular domain binds to laminin-5, an abundant protein in the extracellular matrix. The interaction of

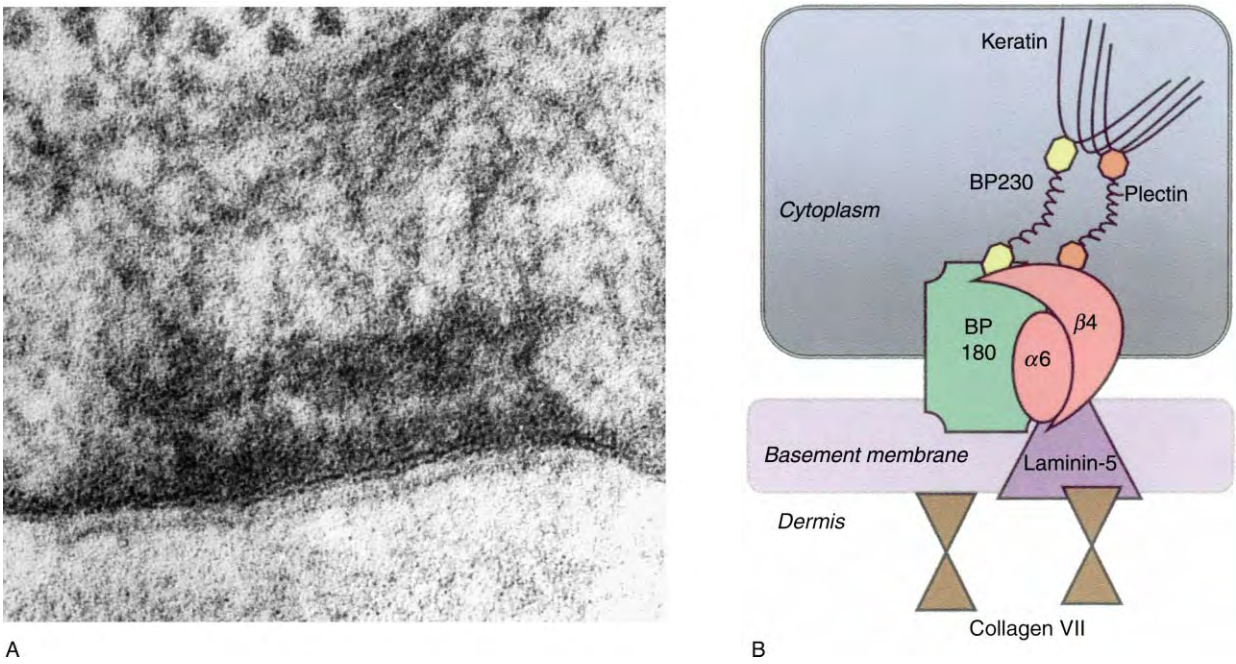



**FIGURE 2** Molecular composition and structure of the hemidesmosome. (A) Hemidesmosome ultrastructure is shown in this electron micrograph of bovine tongue epidermis. Reproduced from Is the hemidesmosome a half desmosome? An immunological comparison of mammalian desmosomes and hemidesmosomes, J. C. R. Jones *et al.*, *Cell Motility and the Cytoskeleton*, Vol. 6, pp. 560–569, Copyright (1986). Reprinted by permission of Wiley-Liss, Inc., subsidiary of John Wiley & Sons, Inc. (B) Hemidesmosomal proteins, their interactions, and their approximate position within the organelle are depicted in this schematic diagram of the hemidesmosome.

$\alpha 6\beta 4$ integrin with laminin-5 is thought to be the structural center of the hemidesmosome, as well as the primary channel for the transduction of hemidesmosome-mediated signaling cascades. The $\beta 4$ subunit interacts with another hemidesmosomal protein, plectin, to anchor the IF cytoskeleton to the hemidesmosome core. This interaction is thought to stabilize the association of $\alpha 6\beta 4$ integrin with laminin-5 to enhance adhesion strength.

## BP180

Bullous pemphigoid antigen II (BP180) is a 180-kDa type II transmembrane protein component of the hemidesmosome, so named because it is recognized by autoantibodies from the serum of patients with the severe blistering disease bullous pemphigoid (Table II). A member of the collagen family, BP180 is a trimer that contains collagen-like repeats in its C-terminal extracellular domain. These repeats organize into a triple helical coiled-coil arrangement thought to contribute to the structure of anchoring filaments. BP180 interacts with $\beta 4$ integrin in the cytoplasm through its N-terminal domain and binds directly to $\alpha 6$ integrin via its C-terminal extracellular domain, an interaction essential for hemidesmosome formation and stabilization. BP180 is also known to interact with actinin family members and codistributes at sites of cell–cell contact with adherens junction proteins, suggesting a potential role for this molecule in mediating cross-talk between cell–substrate and cell–cell junctions.

### BP230

Bullous pemphigoid antigen 230 (BP230) is a 230-kDa cytoplasmic plaque component of the hemidesmosome and a plakin family member. BP230 is known to interact with IFs through its C-terminus. BP230 and BP180 directly interact through their N-terminal domains. BP230 is thought to link the keratin IFs to BP180 and the hemidesmosomal plaque. BP230-null mice have poorly formed hemidesmosomes with few filament attachments.

### Plectin

Plectin, a 500-kDa plakin family member, is particularly important for hemidesmosome function in epithelial tissues and as a cytoplasmic cytoskeletal linker in other tissues, but has also been reported in less abundance in desmosomes. Plectin's importance for hemidesmosome stability and tissue integrity is emphasized in patients with genetic mutations in the plectin gene who suffer from severe skin blistering and

**TABLE II**

**Human Diseases Associated with Hemidesmosomal Components**

| Mutated gene/target antigen | Phenotype |
|---|---|
| **Genetic diseases** | |
| Laminin 5 | *Herlitz or Non-Herlitz junctional epidermolysis bullosa (JEB)*: Autosomal recessive severe blistering at the dermal–epidermal junction often coincident with early mortality (Herlitz); less severe form (non-Herlitz) often includes lifelong blistering, loss of hair and nails, and dental abnormalities |
| $\beta$4 Integrin (missense mutation in cytoplasmic tail) | *Non-Herlitz junctional epidermolysis bullosa (JEB)*: Autosomal recessive severe life-long blistering at the dermal–epidermal junction, together with hair, nail, and teeth defects |
| $\alpha$6 or $\beta$4 Integrin | *Junctional epidermolysis bullosa with pyloric atresia (JEB-PA)*: Mucocutaneous blistering associated with congenital intestinal abnormalities |
| Collagen VII | *Dystrophic epidermolysis bullosa (DEB)*: The most severe form of epidermolysis bullosa characterized by deep blistering in dermis due to disruption of anchoring fibril stability |
| BP180 | *Non-Herlitz JEB/generalized atrophic benign epidermolysis bullosa (GABEB)*: Autosomal recessive generalized skin blistering, nail dystrophy, enamel hypoplasia, and hair loss |
| Plectin | *Epidermolysis bullosa with muscular dystrophy (EB-MD)*: Skin blistering noted from birth with late-onset, progressive muscle weakness |
| **Autoimmune diseases** | |
| Laminin-5 and BP180 | *Cicatricial pemphigoid*: Chronic subepithelial blistering of mucous membranes and occasionally the skin caused by circulating autoantibodies against laminin-5 or BP180 |
| $\alpha$6 and $\beta$4 Integrin | *Ocular cicatricial pemphigoid*: Subepithelial blistering that mainly affects conjunctiva and other mucous membranes caused by circulating autoantibodies against $\alpha$6 and $\beta$4 integrin |
| Collagen VII | *Epidermolysis bullosa acquisita*: Minor skin trauma induces severe blistering below the dermal–epidermal junction caused by circulating autoantibodies against collagen VII |
| BP180 and BP230 | *Bullous pemphigoid*: Generalized large subepidermal blisters caused by circulating autoantibodies against BP180 and BP230 |

muscular dystrophy (Table II). Plectin can interact with itself, with IFs, and with multiple domains of the $\beta$4 integrin tail. Plectin has been proposed to cluster $\alpha6\beta4$ molecules at the basal cell surface, a potentially critical step in the formation of hemidesmosomes. In addition, plectin is required for the ligand-independent assembly of hemidesmosomes. However, the primary role for plectin (like BP230) is to link the IF system to the hemidesmosome plaque, stabilizing the junction by connecting the IF cytoskeleton to the hemidesmosome core at multiple points.

## CD151

CD151, a member of the tetraspan family, is a potential hemidesmosome component. By immunoelectron microscopy, CD151 appears concentrated at hemidesmosomes in an $\alpha6\beta4$ integrin-dependent manner. Although CD151 associates with $\alpha6\beta4$ integrin and may help stabilize and organize the hemidesmosome, the exact nature of CD151 as a hemidesmosomal component requires further investigation.

## Laminin-5

Laminin-5 is a member of the laminin family of large heterotrimeric glycoproteins, which are major constituents of extracellular matrices. Highly expressed in many types of epithelia, laminin-5 is specifically composed of subunits $\alpha3$, $\beta3$, and $\gamma2$, which interact in a noncovalent fashion to form a cross-shaped structure. Laminin-5 is necessary for the firm attachment of basal epithelial cells to the subepithelial basement membrane by linking the hemidesmosome to the underlying tissue. In the hemidesmosome, laminin-5 binds to $\alpha6\beta4$ integrin to mediate cell–substrate attachment. However, laminin-5 is also known to mediate epithelial cell attachment through the nonhemidesmosomal integrin $\alpha3\beta1$. Laminin-5 strongly attaches epithelial cells to the extracellular matrix by interacting with other basement

membrane proteins, including collagen type VII. Laminin-5 can also organize together with BP180 into anchoring filaments thought to extend from the sub-basal dense plate of the hemidesmosome to the deep extracellular matrix. Genetic or autoimmune diseases involving laminin-5 are characterized by complete separation of the epidermis from the dermis (Table II) and further support an important role for laminin-5 in the attachment of basal epithelial cells to the underlying basement membrane.

## Hemidesmosome Assembly and Maintenance

The nucleation and assembly of hemidesmosomes is dependent on the $\alpha6\beta4$ integrin pair and its phosphorylation state. The $\beta4$ integrin tail is believed to play important roles in the initiation of hemidesmosome assembly by inducing early clustering of the $\alpha6\beta4$ integrin pair and recruiting BP180, BP230, and plectin to the nucleation site. The $\alpha3\beta1$ integrin pair is also suspected to be an initiator of hemidesmosome assembly, perhaps by preconfiguring the basement membrane into a conformation conducive for stable hemidesmosome formation.

The BP antigens are also thought to be important players in regulating hemidesmosome assembly. Patients with mutations in the BP180 gene have normal $\alpha6\beta4$ integrin and laminin-5 localization but absent or underdeveloped hemidesmosomes. Also, the overexpression of BP230 in cells alters the localization of endogenous BP180, suggesting a role for BP230 in initiating the early interaction of some hemidesmosome proteins prior to junctional incorporation. Laminin-5 also contributes to the regulation and maintenance of hemidesmosomes. Laminin-5 itself is capable of and essential for initiating the formation of hemidesmosome junctions. Hemidesmosome induction, however, is related to the enzymatic cleavage of the $\alpha3$ laminin-5 subunit. The processing of laminin-5 promotes hemidesmosome formation and discourages motility. For adhesion, laminin-5 appears to initially interact with $\alpha3\beta1$ integrin to mediate the attachment of the epithelial cells to the extracellular matrix. Ligation is then transferred to $\alpha6\beta4$ integrin for long-term stable adhesion. The interaction of laminin-5 with $\alpha6\beta4$ integrin induces the formation of the hemidesmosome core.

## Hemidesmosome Function

Hemidesmosomes were long thought to be inert adhesive structures with primary importance in maintaining and stabilizing epithelial cell attachment to the underlying basement membrane. However, recent evidence suggests that these structures also exhibit dynamic characteristics that may facilitate tissue-remodeling processes during development and wound healing, as well as permitting rapid responses to biological signals.

### Cell–Substrate Adhesive Function

A structural function for hemidesmosomes in initiating and maintaining adhesion between basal epithelial cells and the extracellular matrix is well accepted today, in part because of evidence from autoimmune and genetic human skin diseases that result in epithelial cell-basement membrane separation and severe blister formation (Table II). Other evidence supporting this structural role comes from the noted absence of hemidesmosomes in cells closing wounds and in invasive epithelial tumor cells. Thus, hemidesmosomes appear necessary for stable anchorage of cells to the basement membrane, but may not be required in motile cells.

### Morphogenetic Function

The role that hemidesmosomes play in morphogenetic events such as breast epithelial tubule formation indicates the relative dynamic nature of these structures *in vivo*. Indeed, perturbation of the microfilament cytoskeletal architecture in cultured cells results in a redistribution of IF-tethered hemidesmosomes to the cell periphery, highlighting the dynamic nature of the hemidesmosome plaque and its ability to move laterally within the cell membrane.

### Transmembrane Signaling Function

Hemidesmosomes are also critical signaling centers competent to transduce both outside-in and inside-out signaling events to regulate such cellular activities as gene expression, cell proliferation, and differentiation. Outside-in signaling during ligation of $\alpha6\beta4$ integrin by laminin-5 is known to regulate cell-cycle progression via the Ras/mitogen-activated protein kinase (MAPK) pathway. Although not fully understood, $\alpha6\beta4$ integrin has also been reported to regulate cell survival. Signal transduction through $\alpha6\beta4$ integrin can modulate cell motility characteristics, particularly through phosphorylation of the $\alpha6\beta4$ integrin pair. Inside-out signaling involving $\alpha6\beta4$ integrin is supported by the dephosphorylation of an 80-kDa membrane-bound protein following the disruption of the $\alpha6\beta4$ integrin–laminin-5 interaction, suggesting that signals inside the cell may contribute to the ligation of $\alpha6\beta4$ integrin by laminin-5.

# Conclusion

Despite their ultrastructural and functional similarity as adhesive junctions, desmosomes and hemidesmosomes

are unique structures. Significant progress has been made thus far in the fields of desmosome and hemidesmosome research. The structural organization of these junctions has been examined. Molecular components that make up these junctions have been identified. Protein–protein interactions that contribute to the normal function of these organelles have been characterized. Future studies in these fields will probably focus on further examining the dynamic nature of desmosomes and hemidesmosomes, identifying potential nonadhesive functions for the protein components of these junctional complexes, and investigating junction-mediated signaling cascades and their regulation.

## See Also the Following Articles

Cadherin Signaling • Cadherin-Mediated Cell–Cell Adhesion • Integrin Signaling • Intermediate Filament Linker Proteins: Plectin and BPAG1

## Glossary

**cadherins** Single-pass transmembrane proteins that mediate calcium-dependent cell–cell adhesion.

**epithelia** Sheets of tightly packed cells that cover a body surface or line organs and body cavities.

**extracellular matrix** An organized network of proteins and polysaccharides that fills the extracellular space of tissues and is produced by the surrounding cells.

**integrins** Transmembrane linker proteins that are receptors for extracellular matrix proteins and link the extracellular matrix to components of the cytoskeleton.

**intermediate filaments (IFs)** Cytoskeletal filaments, typically 10 nm in diameter, found in higher eukaryotic cells.

**signal transduction** The propagation of a chemical or mechanical stimulus to effect a cellular response.

## Further Reading

Bannon, L. J., Goldfinger, L. E., Jones, J. C. R., and Green, K. J. (2001). Desmosomes and hemidesmosomes. In *Cell Adhesion*, (M. C. Beckerle, ed.) Vol. 39, pp. 324–368. Oxford University Press, New York.

Borradori, L., and Sonnenberg, A. (1999). Structure and function of hemidesmosomes: More than simple adhesion complexes. *J. Invest. Dermatol.* **112**, 411–418.

Garrod, D. R., Merritt, A. J., and Nie, Z. (2002). Desmosomal cadherins. *Curr. Opin. Cell Biol.* **14**, 537–545.

Getsios, S., Huen, A. C., and Green, K. J. (2004). Working out the strength and flexibility of desmosomes. *Nat. Rev. Mol. Cell Biol.* **5**, 271–281.

Jamora, C., and Fuchs, E. (2002). Intercellular adhesion, signaling, and the cytoskeleton. *Nat. Cell Biol.* **4**, E101–E108.

Jones, J. C. R., Hopkinson, S. B., and Goldfinger, L. E. (1998). Structure and assembly of hemidesmosomes. *Bioessays* **20**, 488–494.

Leung, C. L., Green, K. J., and Liem, R. K. (2002). Plakins: A family of versatile cytolinker proteins. *Trends Cell Biol.* **12**, 37–45.

Nievers, M. G., Schaapveld, R. O., and Sonnenberg, A. (1999). Biology and function of hemidesmosomes. *Matrix Biol.* **18**, 5–17.

Nishiyama, T., Amano, S., Tsunenaga, M., Kadoya, K., Takeda, A., Adachi, E., and Burgeson, R. E. (2000). The importance of laminin-5 in the dermal–epidermal basement membrane. *J. Dermatol. Sci.* **24**(Suppl. 1), s51–S59.

Pulkkinen, L., and Uitto, J. (1999). Mutation analysis and molecular genetics of epidermolysis bullosa. *Matrix Biol.* **18**, 29–42.

## Biography

Rachel L. Dusek is a Ph.D. candidate in the Cancer Biology track of the Integrated Graduate Program and is secondarily affiliated with the Departments of Pathology and Dermatology and the R. H. Lurie Comprehensive Cancer Center at Northwestern University Feinberg School of Medicine. Her primary research interest is in the field of cell–cell adhesion, specifically, the potential nonadhesive functions of the desmosome components.

Jonathan C. R. Jones, Ph.D. is a Professor of Cell and Molecular Biology at Northwestern University Feinberg School of Medicine. He is secondarily affiliated with the Pulmonary Division of the Department of Medicine and the Robert H. Lurie Comprehensive Cancer Center of Northwestern University.

Kathleen J. Green, Ph.D. is the Joseph L. Mayberry Professor of Pathology and a Professor of Dermatology at Northwestern University Feinberg School of Medicine and is affiliated with the Robert H. Lurie Comprehensive Cancer Center of Northwestern University.

# Detergent Properties

**Darrell R. McCaslin**
*University of Wisconsin, Madison, Wisconsin, USA*

**A detergent is formed when a hydrophilic group with an affinity for water and a hydrophobic group with an aversion to water are spatially segregated within a molecule's chemical skeleton so as to create a polarity. In aqueous solution, detergents generally exist as a mixture of monomers in equilibrium with a fairly monodisperse population of detergent aggregates known as micelles. In the micelle, the hydrophobic groups are packed together to create a hydrophobic core with the attached hydrophilic groups projecting out from the surface of this core and protecting it from contact with water. As such, detergents have only limited biological functions such as the well-known role in digestion and possibly to some extent in membrane fusion events; however, they play necessary if not essential roles in the isolation, manipulation, and characterization of the constituents of biological membranes.**

## Chemical Structure of Detergents

The chemical structures of detergents which have been employed in biological studies are quite varied and continues to grow as investigators attempt to develop new entities that will permit the facile isolation of membrane constituents while enhancing their stability once isolated. To reach this goal, various combinations of hydrophobic and hydrophilic groups have been utilized and yield a variety of physical and chemical properties.

### Hydrophobic Groups (Tails)

#### Simple Hydrocarbon Chains

The hydrocarbon chain is the most easily recognized hydrophobic group and when present is often referred to as the molecule's hydrophobic tail (Figure 1). These chains are most often saturated hydrocarbons (Figure 1) and are available in many lengths. The shorter the chain the less well defined the detergent properties tend to be, whereas longer tails become essentially insoluble. Unsaturation and more complex branching structures have been explored as well as chains incorporating phenyl rings. The presence of the ultraviolet light-absorbing phenyl should generally be avoided as it can complicate various spectroscopic techniques.

#### Ring-Based Hydrocarbons

The naturally occurring bile salts synthesized from cholesterol and utilized in digestive processes are the prototypes for detergents based on ring systems. Figure 1 shows cholic acid, one of the abundant bile salts which is converted to deoxycholate (DOC) by removing the hydroxyl at the arrow. Identifying the spatial segregation of the hydrophilic and hydrophobic groups in these detergents may require careful inspection of their three-dimensional chemical structure. In cholic acid, the carboxyl group is an obvious hydrophilic group, but the effects of the hydroxyl groups located on the rings must also be considered. Viewing the steroid ring nucleus in the molecule as defining a plane, the spatial arrangement of the hydroxyls creates a hydrophilic face on one side and a hydrophobic face on the other side of this plane. The structure of DOC suggests the presence of only a hydrophilic edge along the ring plane, making the whole structure more hydrophobic than cholic acid, which may explain why DOC is generally a more aggressive solubilizer than cholic acid.

### Hydrophilic Groups (Headgroups)

#### Ionic

Positively (e.g., amino) and negatively (e.g., carboxyl) charged groups have been utilized as hydrophilic groups. Single-tailed ionic detergents tend to denature all proteins, with the anionic ones being more aggressive at denaturation than cationic molecules. One of the most familiar examples is the anionic detergent, sodium dodecyl sulfate, $NaDodSO_4$ (Figure 1). It is the denaturing effect of $NaDodSO_4$ on proteins that led to the development of one of the most widely exploited analytical tools of biochemistry, denaturing gel electrophoresis, which permits one to easily assess the number of components present in a sample as well as their approximate molecular weights. Interestingly, it is the monomeric form of the ionic detergent that drives the denaturation process.

The bulk of the lipids which form the basic membrane framework are amphiphiles with two hydrophobic

 © 2004, Elsevier Inc. All Rights Reserved. 

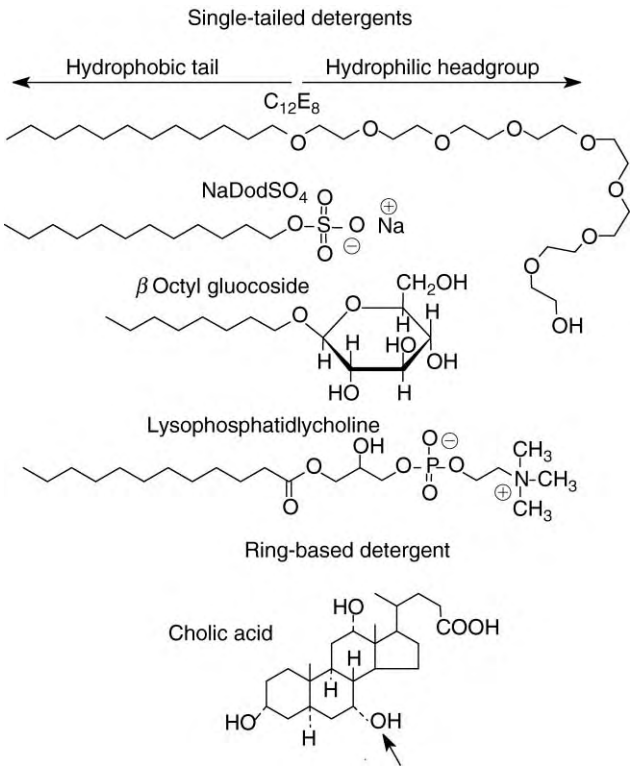



**FIGURE 1** Selected chemical structures of detergents. Removal of the hydroxyl near the arrow on cholic acid coverts the molecule to deoxycholic acid.

tails esterified to zwitterionic headgroups (contains both positive and negative charges). While the lipids themselves generally form vesicular structures when dispersed in water, removal of one of the tails creates a detergent such as the lysophosphatidylcholine in Figure 1. Such lysolipids and other zwitterionic detergents (e.g., sulfobetaines) have been used as well as highly dipolar groups (e.g., amine oxide) that lack a formal charge.

#### Nonionic

Any uncharged group of atoms capable of accepting or donating hydrogen bonds to water can in principle become a nonionic headgroup. The headgroup must be big enough so that the detergent dissolves in solution (as a mixture of monomer and micelles) rather than forming a separate phase. Thus, simple alkyl alcohols tend to phase separate for all but the shortest hydrophobic tails and have no useful detergent properties. Carbohydrate groups are useful headgroups and one of the most frequently encountered is $\beta$-octyl glucoside (OG) in Figure 1. Octyl glucoside in the $\alpha$-anomeric configuration is essentially insoluble, demonstrating how a simple change in chemical structure can dramatically alter detergent properties. More elaborate carbohydrate structures have also been explored.

Commercial applications of detergents have driven the development of a large class of nonionic detergents with polyoxyethylene (POE) headgroups. A chemically homogeneous example is $C_{12}E_8$ (Figure 1), where a 12-C tail ($C_{12}$) is attached to a polymer with eight repeats of $-CH_2CH_2O-$ ($E_8$) and terminates with a hydroxyl group. The ether groups of this chain provide sites for hydrogen bonding with water offsetting the hydrophobic nature of the intervening ethylene groups. Most commercially available POE-based detergents have headgroups consisting of a single chain although the length is often heterogeneous; however, highly branched structures have been created by attachment of several POE chains to a central moiety such as sorbitol (e.g., Tweens). The POE chains are subject to peroxidation and breakdown and solutions should only be used when relatively freshly prepared; moreover, when using commercial sources one should be aware that antioxidants are sometimes included.

### Connecting Head and Tail

Linkages between the hydrophobic and hydrophilic groups are most often either an ether or ester linkage. The latter can be susceptible to hydrolysis, especially in biological preparations, which in turn could generate an ionic group with denaturing properties.

## Detergent Properties

### Critical Micelle Concentration (CMC)

The most common concentration-dependent behavior for detergents is illustrated in Figure 2. The monomer concentration increases until the critical micelle concentration (CMC) above which an equilibrium is established between the monomer and an increasing concentration of micelles. The monomer concentration increases very little after reaching the CMC and thus is in effect the highest possible concentration of monomeric detergent. All detergent in excess of the CMC is incorporated into micelles (dashed line in Figure 2). The concentration of micelles increases linearly with total detergent concentration above the CMC, but at a slower rate since a large number of molecules are incorporated into each micelle. Some detergents exhibit more complex behavior, such as secondary association of micelles or even new phases as the total concentration continues to increase.

As illustrated in Figure 2, the CMC is actually a narrow range of concentration over which the formation of micelles becomes dominant. Nonetheless, a single number is usually defined as the CMC and determined by plotting some physical observable whose response differs markedly above and below the CMC against the total

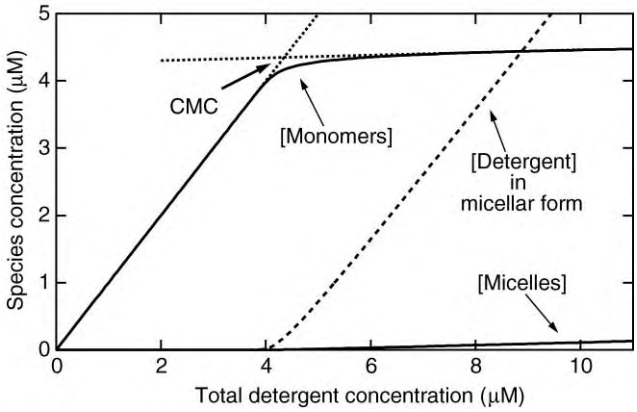



**FIGURE 2** Concentration-dependent behavior of a detergent. The calculations are based on the theory of micelle formation developed by Tanford. An aggregation number of 50 was used and the equilibrium constant for micellization was chosen to yield the critical micelle concentration (CMC) indicated.

concentration of detergent. The CMC is then taken as the intersection of lines extrapolated from the nearly linear regions above and below the transition as shown by the dotted lines in Figure 2. Using different observables or concentration ranges for the extrapolation can lead to somewhat different values for the CMC. One of the simplest methods to measure a CMC concentration ranges or for the extrapolation. is by solubilization of heme, which is essentially insoluble below the CMC and increases as the concentration of micelles increases and would be similar to the dashed line in Figure 2.

CMCs range from nanomolar to several millimolar. For single-tailed detergents, the CMC decreases with increasing length of tail and with smaller headgroups. The CMC of nonionics is generally lower than that of ionics with the same length of tail. Ionic headgroups are more susceptible to solution variables than the nonionics. Ionic headgroups that can be titrated are sensitive to pH and may precipitate when the headgroup is in neutral form (both cholic acid and DOC are examples). The CMCs of ionics are also influenced by the type and concentration of counterions present, e.g., using potassium instead of sodium for dodecyl sulfate, results in an insoluble salt. In general, higher salt concentrations will decrease the CMC of an ionic detergent which is a consequence of reducing the headgroup repulsion in the micelle. Even the POE-based nonioncs may be influenced by presence of salts as the ether groups are capable of complexing ions. The solubilization of other components in the micelle (e.g., mixed micelles with lipids) will generally decrease the CMC (i.e., the maximum monomer detergent concentration is decreased). Published values of CMCs are useful guides in experimental design, but should generally be confirmed under the actual experimental conditions.

## Aggregation Number

Micelles are formed by self-association of many detergent molecules into a single noncovalent structure. Since the number of molecules in each micelle is somewhat variable, micelle size is characterized by the aggregation number, which is the average number of molecules per micelle and can be determined by standard hydrodynamic methods (e.g., sedimentation equilibrium). The aggregation number is needed to calculate the micelle concentration, which is the total detergent concentration in excess of the CMC divided by the aggregation number.

The CMC transition becomes sharper as the aggregation number increases. The CMC is generally strongly dependent on the size of the hydrophobic moiety, but repulsion between headgroups is the dominant factor for the aggregation number. Thus, nonionics tend to have larger aggregation numbers than ionics of the same tail length. The aggregation number for ionic detergents can be strongly influenced by both the type and concentration of counterions present as well as pH since these factors can dramatically change the electrostatic repulsion between the charged headgroups. Longer hydrophobic tails tend to have larger aggregation numbers. Detergents with high CMCs tend to have more variability in their micelle size distribution. At high concentrations and under certain solution conditions (e.g., elevated temperature) larger structures may be formed which in some cases are new micellar phases and in others simply secondary aggregation of smaller micelles.

## Temperature Effects

As a thermodynamic equilibrium, the formation of micelles can be influenced by temperature. At low enough temperatures, solid detergent will exist in equilibrium with monomeric detergent; as the temperature increases, the monomer concentration increases until it reaches the CMC at the critical micelle temperature. Above this temperature, solid detergent will begin to go into solution as micelles. The temperature at which solid, micelles, and monomer at the CMC coexist is called the Kraft point and for most detergents is the same as the critical micelle temperature. An often observed Kraft point, which is near room temperature, is that of sodium dodecyl sulfate and upon cooling one sees precipitation of detergent. A second temperature effect observed at higher temperature, especially with nonionics containing POE, is the cloud point. At this temperature and above the CMC, the solution will turn turbid due to the formation of much larger aggregates. Both the critical micelle temperature and cloud point have been exploited in purification of membrane components.

## Micelle Structure

### Single-Tailed Detergents

In a micelle, the hydrophobic tails are sequestered into a central core structure with the hydrophilic groups projecting out from the surface of this core into the aqueous surrounding. The tails in the core are not fully extended but are quite flexible, some even lying along the surface of the core rather than within it. The surface of the core should be regarded as having a rippled texture with some tail methylene groups protruding above it. Experimental and theoretical arguments suggest that for most detergent micelles the overall shape is best described as an oblate ellipsoid, although for small aggregation numbers the shape cannot be readily distinguished from spherical. For $C_{12}E_8$ micelles (Figure 1), theoretical calculations constrained by hydrodynamic measurements yield an oblate ellipsoid with dimensions shown in Figure 3. One dimension must be less than the 3.4 nm length of two fully extended 12C chains since the core cannot contain a void. For the same length of tail, the overall dimensions of the micelle will depend on the nature of the headgroup. For POE-based detergents, the headgroup is in a random-coil configuration and consequently occupies a very large region of the space surrounding the core, as illustrated in Figure 3.

Figure 3, while a convenient visualization of micelle structure, cannot convey the highly dynamic processes occurring. Detergent molecules are rapidly exchanged among micelles; whole micelles disappear and reform with slightly different aggregation numbers. The hydrophobic core acts for the most part like a simple liquid hydrocarbon with the tails constantly flexing. POE headgroups occupy a great deal of space around the core and are constantly changing their conformation as well. Finally, it should be obvious that the type and size of headgroup can generate dramatically different chemical environments to which molecules solubilized by detergents are exposed; moreover, the solution composition near a micelle may be quite different from that of the bulk aqueous solution.

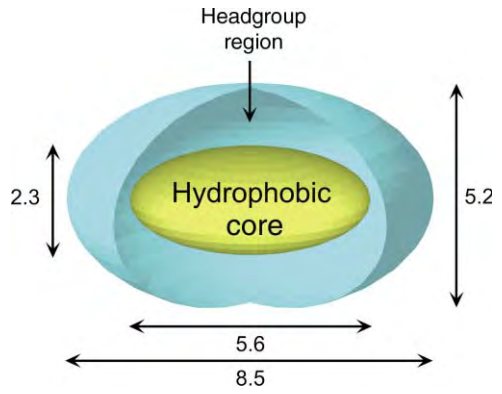



**FIGURE 3** Shape of micelles formed by typical single-tailed detergents. The model shown is based on data and calculations for C12E8. The dimensions shown are in nm.

### Ring-Based Detergents

Bile salts usually have relatively high CMCs and quite small aggregation numbers (4–10). The small aggregation number does not permit micelles as just described. The micelles are more heterogeneous and are probably best viewed as small assemblies with the hydrophobic surfaces facing each other, presenting their hydrophilic faces to the surrounding solution. The size and shape of these micelles are quite sensitive to the concentration and types of ions, and potentially pH.

## Detergents as Tools in Membrane Studies

### Membrane Solubilization

Initially when a membrane is exposed to a detergent, monomers partition into the bilayer, so one can imagine that a high CMC might be advantageous (e.g., OG has a very high CMC (>15 mM) and is an efficient solubilizer). As more detergent intercalates into the membrane's bilayer, one reaches a stage where fragmentation occurs, resulting in large mixed micelles containing detergent, lipids, and proteins in various ratios. Continuing to add detergent will eventually disperse the membrane components to the point that any given micelle contains no more than a few lipids, a protein, or the detergent alone. One can apply isolation and enrichment techniques for specific targets to this solubilized mix. The choice of detergent can hinder or help in isolation, e.g., a small aggregation number can facilitate separation of a large protein-containing micelle from those containing only lipid components. It is possible, and perhaps even desirable, to exchange detergents, so that one detergent might be used to speed initial membrane solubilization and another for final isolation of a stable target. It is important to recognize that it is the concentration of micelles that needs to be controlled when one is studying membrane components solubilized by detergents, and generally one should maintain a ratio of several micelles to each solubilized component to avoid the possibility of spurious associations.

### Membrane Proteins

A detergent can be found that can reasonably mimic the hydrophobic core of the native membrane. However, the membrane provides a physical constraint to the embedded protein, in that the protein cannot expand

laterally in the plane of the membrane nor can it pull itself through the bilayer; in a detergent solution, such a physical constraint is greatly relaxed and the protein in effect has a much expanded conformational space including conformations where activity will be lost possibly irreversibly. Low CMCs and large aggregation numbers may partially compensate for the loss of the membrane's physical constraints. Moreover, the native environment of the membrane provides a multitude of interactions with a variety of lipid headgroups, tails, and with other proteins and these interactions may differ from one side of the membrane to the other. The roles of these interactions in maintaining an active protein are not well understood and they are radically changed upon solubilization. Studies as a function of micelle concentration should be performed, as there is always a possibility of adventitious interactions, due merely to the fact that too little detergent is present.

## Reconstitution of Protein into a Bilayer

Many of the functions of membrane proteins are vectorial in nature, and one must eventually reconstitute the protein into a lipid bilayer where these functions can be probed. Starting with mixed micelles of protein in detergent and lipids in detergent, one must effect a controlled removal of the detergent, permitting the formation of an artificial membrane bearing the protein. This is accomplished largely by trial and error. A high CMC permits the use of dialysis since the monomer should pass through the dialysis membrane. Hydrophobic beads to which the detergent absorbs have also proven effective in reconstitution studies.

## Summary

Obviously single-tailed ionic detergents should generally be avoided because of their potential to denature any protein. While zwitterionic headgroups can be identical or at least similar to those of native lipids, to date they have not proven any more useful than others. High CMCs may be appropriate choices for initial solubilization and eventual reconstitution. But long tails and large headgroups (usually accompanied by a low CMC) may enhance stability by restricting the conformational space accessible to the protein in detergent solution. The requirements for efficient solubilization and for the maintenance of activity may to a large extent be antagonistic. The possibility of using one detergent to solubilize and subsequently exchanging into another to enhance stability for isolation and study should always be considered. Finally, there is no perfect detergent for all situations nor are there hard-and-fast rules for choosing a detergent for a specific task. In the end the choice is still largely a matter of trial and error.

## See Also the Following Articles

MDR Membrane Proteins • Membrane Transport, General Concepts

## Glossary

**aggregation number** Number of detergent molecules in the average micelle.

**CMC (critical micelle concentration)** Concentration of detergent in aqueous solution above which micelles begin to form, essentially the maximum concentration of detergent in monomeric form.

**detergent** A compound having spatially segregated hydrophilic and hydrophobic regions and which, when dissolved in water above the CMC, self-associates to form micelles.

**hydrophilic group** Structure having an affinity for water with strong favorable noncovalent bonds with water molecules.

**hydrophobic group** Structure exhibiting an aversion to water and preference for hydrocarbon-type liquids.

**micelle** Structure formed by the noncovalent and highly cooperative self-association of a detergent molecule so as to form a hydrophobic core from which the hydrophilic groups project into the aqueous surroundings.

## Further Reading

Gravito, R. M., and Ferguson-Miller, S. (2001). Detergents as tools in membrane biochemistry. *J. Biol. Chem.* **276**, 32403–32406.

Helenius, A., and Simons, K. (1975). Solubilization of membranes by detergents. *Biochim. Biophys. Acta* **415**, 29–79.

Helenius, A., McCaslin, D. R., Fries, E., and Tanford, C. (1979). Properties of detergents. *Methods Enzymol.* **56**, 734–749.

Tanford, C. (1980). *The Hydrophobic Effect: Formation of Micelles and Biological Membrane.* Wiley, New York.

Tanford, C., and Reynolds, J. A. (1976). Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta* **457**, 133–170.

White, S. H., Ladokhin, A. S., Jayasinghe, S., and Hristova, K. (2001). How membranes shape protein structure. *J. Biol. Chem.* **276**, 32395–32398.

## Biography

Dr. Darrell R. McCaslin holds a B.S. with Honors in biochemistry from Oklahoma State University and a Ph.D. in physical biochemistry from Duke University. He has held positions at Duke University Medical Center and Rutgers University in Newark. Presently, he is the Director of Operations for the Biophysical Instrument Facility at the University of Wisconsin in Madison. In addition to overseeing the operations of the facility and training investigators in the use of the instrumentation, he collaborates on a variety of characterization problems.

# Diabetes

**David W. Cooke**
*The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA*

**Diabetes mellitus is among the most common chronic diseases, affecting over 6% of the adult population in Western society, and with a prevalence that is rising dramatically worldwide. However, diabetes mellitus is not a single disease, but rather a number of distinct disorders. They share, in common, deficient action of the hormone insulin. This can be due to an absolute deficiency of insulin production and secretion by the $\beta$-cells of the pancreatic Islets of Langerhans. However, in many types o diabetes mellitus there is an impaired cellular response to insulin; this insulin resistance imposes a requirement for increased insulin levels to maintain metabolic control. In this case, diabetes mellitus occurs when there is an inability to secrete sufficient insulin to meet this increased demand. The insulin deficiency, whether absolute or relative, leads to a failure to control carbohydrate and lipid metabolism.**

## Introduction

The defining feature of diabetes mellitus is an abnormal elevation of the blood glucose level. When the degree of hyperglycemia is mild, there may be no symptoms of diabetes. With greater degrees of hyperglycemia, the concentration of glucose in the blood exceeds the ability of the kidneys to completely reabsorb the glucose delivered to it. The resultant loss of glucose in the urine provides an osmotic force that draws increased water into the excreted urine, causing the patient to experience polyuria (increased urination; this glucosuria gives diabetes mellitus its name, which can be translated from the Greek as "sweet urine" – or more directly as "honey siphon". The osmotic diuresis from glucosuria induces polydipsia (increased drinking) in order to prevent dehydration. When uncontrolled, diabetes mellitus puts patients with these diseases at risk of acute metabolic decompensation, particularly when the insulin deficiency is complete, as in type 1 diabetes mellitus. While this decompensation is life threatening, since the introduction of insulin treatment over 80 years ago these events are generally preventable. It is now the long-term complications that all patients with diabetes mellitus are at risk that has become the most significant burden. Due to these long-term complications, diabetes is a leading cause of blindness and end-stage renal disease. In addition, diabetes is associated with accelerated atherosclerotic vascular disease, leading to a risk of myocardial infarction, stroke, and limb amputation that is increased and occurs at a younger age than in those without diabetes.

## Etiology

For each type of diabetes mellitus, the relative contribution of a defect in insulin secretion versus a defect in insulin action will vary. For example, the two most common forms of diabetes are type 1, which is due to an isolated deficiency in insulin, and type 2, which is due to varying degrees of insulin resistance and deficient insulin secretion. In addition, some forms of diabetes have a predominantly genetic etiology, such as the autosomal dominantly inherited maturity-onset diabetes of the young (MODY), whereas in other types both genetic and environmental factors are important, exemplified by type 2 diabetes, where the development of obesity is a significant contributor to the cause of insulin resistance that is central to the pathophysiology of this disease.

### Type 1 Diabetes Mellitus

Type 1 diabetes accounts for ~5–10% of all cases of diabetes. It typically has its onset in childhood, although it can occur at any age. Type 1 diabetes is due to the destruction of the insulin-producing $\beta$-cells of the pancreas, resulting in an absolute deficiency of insulin. In the vast majority of cases, the destruction is due to a cell-mediated autoimmune attack of the $\beta$-cells. This process appears to be precipitated by environmental factors in genetically susceptible individuals. While viral infections, dietary components and specific toxins have been suspected, the true identity of the environmental triggers remains unknown. Similarly, while the immune-recognition genes of the HLA locus account for much of the genetic risk, there are other unidentified genes that also contribute to this risk.

In almost all cases of type 1 diabetes, there is ultimately complete destruction of the pancreatic

 © 2004, Elsevier Inc. All Rights Reserved. 

$\beta$-cells. Because of this, these patients are dependent on treatment with exogenous insulin for their survival, as the absence of such treatment leads to rapid metabolic deterioration into diabetic ketoacidosis and death.

## Type 2 Diabetes Mellitus

Over 90% of diabetes is due to type 2 diabetes. The prevalence of this disease increases with age; previously it had been uncommon for children or young adults to be diagnosed with this disease. However, there has been a recent dramatic increase in the prevalence of this disease, and with this, significant numbers of children are now being diagnosed with type 2 diabetes. Although much has been learned about the pathophysiology of type 2 diabetes in the past few decades, there remains much to be learnt about the specific details of its cause. What is known is that two defects are present in patients with type 2 diabetes: insulin resistance and defective insulin secretion. Insulin resistance refers to a decreased effectiveness of insulin in activating signals distal to binding of insulin to the insulin receptor. Insulin resistance itself, except in the most extreme situation, will not lead to diabetes, as normal metabolic control can be maintained by a compensatory increase in insulin secretion. When a second defect results in an inability to respond to the requirement for increased insulin secretion imposed by insulin resistance, type 2 diabetes mellitus occurs.

There is strong familial clustering of type 2 diabetes, and the risk for this disease varies considerably across different ethnic populations. These and other findings indicate that there is a strong genetic component to the development of type 2 diabetes. Multiple genes are most likely to be involved, with each individual with diabetes having altered function of multiple gene products. In addition, the relative contribution of insulin resistance versus defective insulin secretion can vary from patient to patient, so that there is likely a subset of altered genes contributing to the development of type 2 diabetes that differs from one person to the next. The genes involved will include those encoding proteins affecting insulin signaling and insulin secretion, those controlling metabolic pathways, and those contributing to the development of obesity.

Environmental factors play a significant role in determining which genetically predisposed individuals will develop type 2 diabetes. Obesity and low levels of physical activity are strongly associated with insulin resistance, and are the factors to which the rising prevalence of type 2 diabetes in industrialized societies are ascribed. In addition, insulin sensitivity (the inverse of insulin resistance) decreases with age, accounting for much of the increased prevalence of this disease with increasing age.

### Insulin Resistance

Both central (hepatic) and peripheral (muscle and adipose) defects contribute to the insulin resistance of type 2 and obesity. (Interestingly, experiments in rodents have indicated that resistance in cells not classically characterized as being insulin-responsive, including the brain and pancreatic $\beta$-cells, may contribute to the pathophysiology of obesity and type 2 diabetes.) Hepatic insulin resistance results in increased glucose output from the liver, predominantly due to increased gluconeogenesis. Insulin-resistant muscle and adipose cells have diminished insulin-stimulated glucose uptake, and the muscle cells have a marked decrease in glycogen synthesis. These defects, particularly in the muscle, result in decreased disposal of glucose after a meal. In addition, the decreased metabolism of glucose to glycogen leads to increased glycolysis and production of lactate, which circulates to the liver and provides substrate for increased gluconeogenesis. The increased hepatic glucose production contributes most to fasting hyperglycemia, while the decreased glucose disposal into muscle contributes most to postprandial hyperglycemia.

Although the mechanisms remain incompletely understood, a number of factors have been demonstrated to contribute to the insulin resistance of type 2 diabetes, including free fatty acids and other factors secreted by adipocytes, as well as hyperglycemia itself (Figure 1). Free fatty acids impair insulin sensitivity, both in the liver and in muscle, and increased circulating levels of free fatty acids are found in obese and insulin-resistant subjects, due to increased lipolysis in adipocytes. Importantly, intra-abdominal fat tissue has a higher lipolytic rate compared to subcutaneous adipose tissue, and excess intra-abdominal adiposity has a much higher association with insulin resistance than obesity with a more peripheral (subcutaneous) distribution. Abnormal secretion by the adipocyte of a number of other factors (adipokines) has also been implicated in

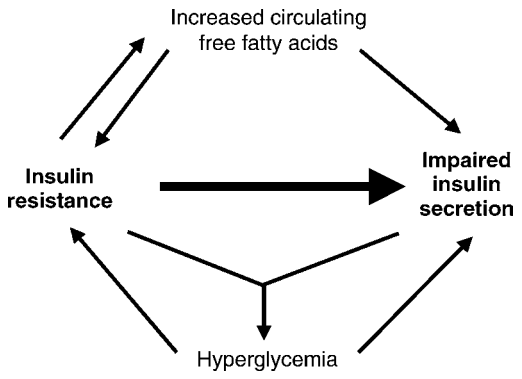
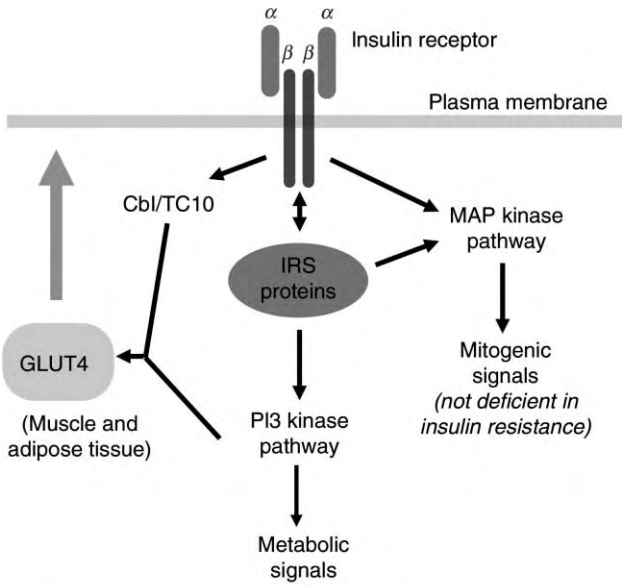



**FIGURE 1** Factors contributing to insulin resistance and impaired insulin secretion include the ability for each of them to affect both themselves and each other through direct and indirect mechanisms.

the pathophysiology of insulin resistance, including TNF-$\alpha$, resistin, adiponectin, and leptin. Hyperglycemia itself causes impaired insulin sensitivity. This may be due to activation of protein kinase C (PKC) by hyperglycemia. Recently, it has also been suggested that hyperglycemia-induced insulin resistance is in some way caused by increased flux of glucose through the glucosamine pathway.

***Molecular Mechanisms of Insulin Resistance*** Insulin is a peptide hormone that binds to a transmembrane-spanning cell surface receptor (Figure 2). The insulin receptor is heterotetrameric in structure, containing two $\alpha$- and two $\beta$-subunits. Insulin binds to the extracellular $\alpha$-subunit, inducing a conformational change in the transmembrane $\beta$-subunit that derepresses intrinsic tyrosine kinase activity contained in the intracellular domain. The activated $\beta$-subunit tyrosine kinase activity first trans-phosphorylates the opposite $\beta$-subunit of the $\alpha_2\beta_2$ receptor complex. Once autophosphorylated, the insulin receptor is capable of phosphorylating intracellular proteins that propagate the insulin signal within the cell. Proteins of the insulin receptor substrate (IRS) family, the most important of these being IRS-1 and IRS-2, recognize and bind to phosphotyrosine motifs on the activated insulin receptor. Once bound, the IRS proteins are themselves phosphorylated on multiple tyrosine residues by the activated insulin receptor. The IRS proteins do not have intrinsic enzymatic activity, but serve as docking sites to bring other proteins into proximity to the insulin receptor. A signaling cascade is initiated, as proteins recognize and bind to the phosphotyrosine motifs on either the insulin receptor or the IRS proteins. This results in their activation, often through their phosphorylation by the insulin receptor.




**Abnormalities found in insulin resistance:**

**Insulin receptor:**
- decreased number (minor)
- decreased insulin-stimulated tyrosine phosphorylation
- increased serine phosphorylation

**IRS proteins:**
- decreased protein level
- decreased insulin-stimulated tyrosine phosphorylation
- increased serine phosphorylation
- increased frequency of IRS-1 polymorphisms (some populations)

**Decreased insulin-stimulated PI3 kinase associated with IRS proteins**

**GLUT4:**
- decreased expression (adipose tissue)
- decreased insulin-stimulated translocation to the plasma membrane (muscle and adipose)

**FIGURE 2** Major signaling pathways of insulin action and abnormalities found in insulin resistance.

The downstream components of the insulin-signaling cascade include protein kinases and phosphatases, and lipid kinases. The two pathways that have been investigated in the most detail are the mitogen-activated protein (MAP) kinase and the phosphatidylinositol-3-OH (PI3) kinase pathways. Although the distinction is not absolute, activation of the MAP kinase pathway is most responsible for the mitogenic actions of insulin, while activation of the PI3 kinase pathway is predominantly involved in the metabolic actions of insulin. This is significant, because activation of the MAP kinase pathway by insulin is not reduced in type 2 diabetes, a result that may be involved in the pathogenesis of the long-term complications of insulin resistance and diabetes. A third pathway involves phosphorylation and activation of the Cbl proto-oncogene by the insulin receptor, which results in activation of the G protein TC10. Many of the pathways that are activated by the insulin receptor are also activated by other means, particularly activation of other growth factor receptors. Specificity of the insulin signal is felt to be due to the integration of the multiple pathways activated, as well as to compartmentalization of the components of the cascade. For example, both the PI3 kinase and Cbl/TC10 pathways appear to be necessary for insulin-stimulated glucose uptake into muscle and adipose cells.

Abnormalities of each of the components of the insulin-signaling cascade, from the insulin receptor at the beginning to the enzymes, transcription factors, and other effector proteins at the end, are potential candidates for contributing to the insulin resistance of type 2 diabetes. Indeed, certain polymorphisms in the IRS-1 gene are found more frequently in individuals with diabetes in some populations (the lack of this association in other populations likely underscores the genetic heterogeneity of this disease). For all the other components, however, mutations or significant alterations in expression have either not been found or have been extremely rare. However, there have been clearly

identified abnormalities in insulin signaling that occur in the insulin resistance of type 2 diabetes (Figure 2). Notably, insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS proteins is decreased in tissues in insulin-resistant subjects. This is often associated with an increase in phosphorylation on serine residues in these proteins, which may inhibit the protein–protein interactions necessary for proper signaling. Potential mechanisms for this include serine-phosphorylation of the insulin receptor by PKC, and stimulation of serine-phosphorylation of IRS-1 by TNF-$\alpha$; PKC activity is increased in the presence of hyperglycemia, and adipocytes from obese subjects express increased amounts of TNF-$\alpha$. The increase in free fatty acids present in obesity and insulin resistance may also lead to increased serine phosphorylation of the insulin receptor and IRS proteins.

In order to enter a cell, glucose requires specialized transporters to be present on the cell surface. GLUT4, the insulin-responsive glucose transporter, is sequestered in intracellular vesicles in the basal state. When muscle or adipose cells are stimulated by insulin, these vesicles translocate and fuse with the plasma membrane, and the increase in transporters at the cell surface results in increased glucose uptake. In the insulin resistance of obesity and type 2 diabetes, there is a decrease in the insulin-stimulated translocation of GLUT4. There may also be a decrease in the intrinsic activity of GLUT4 in insulin-resistant states. Finally, in adipocytes (but not muscle cells) there is significantly decreased expression of GLUT4 that contributes to the diminished insulin-stimulated glucose uptake. Although quantitatively adipose tissue contributes only a small fraction to glucose disposal compared to muscle, evidence from animal models with tissue-specific insulin resistance demonstrates that insulin resistance in one tissue can affect insulin sensitivity in other tissues. Thus, insulin resistance in the adipocyte can cause insulin resistance in liver and muscle, most likely through secreted free fatty acids and adipokines.

### Insulin Secretory Defect

Insulin secretion increases to compensate for insulin resistance, so that in obese or otherwise insulin-resistant individuals, fasting and meal-stimulated insulin levels are elevated. When insulin secretion fails to fully compensate for the degree of insulin resistance, glucose levels rise, first to those of mild glucose intolerance (glucose levels above normal but below criteria for a diagnosis of diabetes), and then to those of diabetes. Insulin levels early in this progression are still elevated compared to individuals with normal insulin sensitivity, but are not elevated sufficient to maintain normal glucose control. With time, in most patients, the insulin secretory defect continues to worsen, including loss of $\beta$-cells, ultimately requiring exogenous insulin treatment to maintain control of blood glucose levels.

As for insulin resistance, the development of impaired insulin secretion is likely due to both genetic and environmental factors. The persistent stimulus imposed by insulin resistance on the $\beta$-cell to oversecrete insulin appears to be a key factor contributing to the loss of $\beta$-cell function (Figure 1). This may be due in part to the effect of excessive secretion of islet amyloid polypeptide (IAPP), which is cosecreted with insulin; in the islets in the majority of patients with type 2 diabetes there is the accumulation of amyloid, containing IAPP. Another factor is the elevated free fatty acid level present in insulin resistance, which can contribute to the defective insulin secretion, just as it can contribute to insulin resistance. While short-term exposure to lipids increases insulin secretion, long-term exposure impairs $\beta$-cell function, and may be responsible for $\beta$-cell death by apoptosis. Similarly, prolonged exposure to increased glucose levels imposes a "glucotoxicity" on the $\beta$-cell: if glucose levels are normalized in a patient with diabetes, endogenous insulin secretion will improve. Over time, however, this glucotoxicity may lead to irreversible $\beta$-cell damage, perhaps through oxidative injury. Finally, insulin resistance of the $\beta$-cell may itself lead to impaired $\beta$-cell function, as demonstrated in mice carrying genetic defects of insulin signaling in $\beta$-cells.

## Other Specific Types

Diabetes of types other than type 1 or type 2 make up a small percentage of the cases of diabetes. For a number of these, however, a single gene defect results in the disease, providing potential insight into what may be the underlying cause of the insulin resistance or insulin secretory defect in type 2 diabetes.

### Genetic Defects of $\beta$-Cell Function

***Insulin Gene*** Mutations in the insulin gene have been described, but are extremely rare causes of diabetes. In the few families where such mutations have been found, the result is very mildly abnormal glucose control, inherited in an autosomal-dominant manner.

***Maturity-Onset Diabetes of the Young*** Maturity-onset diabetes of the young are a group of diabetes types caused by mutations in single genes. Mutations in six genes have been identified, each of which results in deficient insulin secretion. These mutations result in autosomal dominantly inherited forms of diabetes that have an early onset, usually before the age 25. Because these patients retain some insulin secretion, albeit insufficient to prevent hyperglycemia, these patients are, in general, not ketosis prone, and so are not dependent on insulin treatment for survival. In contrast

to type 2 diabetes, obesity and insulin resistance are not a major feature of MODY; the diabetes in these cases is due to the defective insulin secretion. The severity of the insulin deficiency varies for the different genes involved, and consequently the severity of the untreated diabetes can vary from mild, sometimes unrecognized hyperglycemia with little risk of long-term microvascular diabetic complications, to severe hyperglycemia with a very high risk of microvascular complications.

MODY2 is caused by mutations in the gene for glucokinase, which is expressed in highest levels in the pancreatic $\beta$-cell and in the liver. In the $\beta$-cell, glucokinase catalyzes the phosphorylation of glucose to generate glucose-6-phosphate, the first step in glucose metabolism. Because glucose metabolism is the stimulus for insulin release, glucokinase functions as the glucose sensor in the $\beta$-cell as it is rate limiting for glucose entry into the glycolytic pathway. Thus, glucokinase mutations lead to decreased glucose-stimulated insulin secretion. In the liver, glucokinase affects the storage of glucose as glycogen; this defect may contribute to the hyperglycemia in patients with glucokinase mutations. MODY2 is caused by heterozygous mutations of the glucokinase gene; complete glucokinase deficiency due to homozygous mutations lead to more severe neonatal-onset diabetes.

The other genes that have been identified as having mutations that cause MODY are each transcription factors expressed in the pancreatic $\beta$-cell: hepatocyte nuclear factor (HNF)-4$\alpha$ (MODY1); TCF1, which encodes HNF-1$\alpha$ (MODY3); insulin promoter factor-1 (IPF1;MODY4); TCF2, which encodes HNF-1$\beta$ (MODY5); and neurogenic differentiation 1/$\beta$-cell E-box transactivator 2 (NeuroD1/BETA2;MODY6). Each of these genes are expressed in the $\beta$-cell and regulate expression of the insulin gene and genes for other factors involved in the regulation of insulin signaling; heterozygous mutations lead to $\beta$-cell dysfunction sufficient to cause diabetes. IPF-1 and NeuroD1/BETA2 are also involved in pancreatic development, and homozygous mutations of IPF-1 lead to pancreatic agenesis and congenital diabetes mellitus and pancreatic insufficiency.

*Mitochondrial DNA Mutations* Mutations in the mitochondrial genome can cause diabetes mellitus, the most common involving the mitochondrial $tRNA^{LEU(UUR)}$. Due to the maternal origin of mitochondria, this type of diabetes is maternally inherited. The pancreatic $\beta$-cell is rich in mitochondria and has a high rate of oxidative metabolism; the mitochondrial mutations result in a $\beta$-cell defect that causes diabetes through deficient insulin secretion. This form of diabetes is frequently associated with neurosensory deafness.

While inherited mitochondrial genome mutations are rare as a cause of diabetes mellitus, they may provide insight into the worsening insulin secretory defect that occurs in type 2 diabetes. The mitochondrial genome consists only of coding sequences, and its repair mechanisms are poor so that it is highly susceptible to mutation. With age, there is an increase in reactive oxygen species generated in mitochondria, and this may be exacerbated in $\beta$-cells with a higher rate of oxidative metabolism due to exposure to elevated glucose levels. With a low expression of enzymes that defend against oxidative damage, $\beta$-cells are particularly sensitive to oxidative injury. The inherited mitochondrial genome mutations that cause diabetes demonstrate that these mutations can impair insulin secretion, and it is possible that an accumulation of mitochondrial DNA mutations due to oxidative damage contribute to the $\beta$-cell dysfunction of type 2 diabetes.

### Genetic Defects in Insulin Action

*Insulin Receptor Mutations* Insulin resistance can be caused by mutations of the insulin receptor, although these are rare as a cause of diabetes. Most of the subjects identified with insulin resistance and insulin receptor mutations have mutations of both alleles. Some cases of mild insulin resistance have been described in which only one allele carries a mutation, acting as a dominant negative inhibitor of the wild-type allele, due to their combination within the heterotetrameric structure of the receptor. The severity of the phenotype with two mutant insulin receptor alleles can vary from insulin resistance with relatively normal glucose control (type A insulin resistance) to a more severe phenotype evident in infancy (Rabson–Mendenhall syndrome and Leprechaunism, which is usually fatal in infancy).

### Secondary Diabetes Mellitus

A number of systemic disorders can cause marked insulin resistance, which can lead to secondary diabetes mellitus (although as for type 2 diabetes and as discussed above, there must be some defect in insulin secretion to prevent complete compensation for the insulin resistance by the $\beta$-cell). Excess growth hormone, as in acromegaly, excess glucocorticoid, as in Cushing's disease or exogenous glucocorticoid treatment, or excess catecholamines, as in pheochomacytoma, as well as other diseases such as uremia and hepatic cirrhosis can lead to secondary diabetes primarily through their effect on insulin sensitivity. Similarly, diabetes mellitus will occur if a disorder produces sufficient $\beta$-cell damage. Examples of this include pancreatitis, cystic fibrosis, pancreatectomy, and hemochromatosis; in addition, somatostatin-secreting tumors can inhibit insulin secretion sufficient to cause diabetes mellitus.

Rarely, patients with autoimmune disease have been identified where antibodies against the insulin receptor

block signaling, leading to an insulin resistant form of diabetes referred to as type B insulin resistance.

Strikingly, just as the excess adipose tissue in obesity leads to insulin resistance, the deficiency of adipose tissue that is found in lipoatrophy/lipodystrophy also causes insulin resistance, often severe. Mice that are genetically engineered to have a paucity of adipose tissue are also markedly insulin resistant, confirming this association. The cause of insulin resistance in syndromes of adipose deficiency is likely due to intrahepatic and intramuscular accumulation of triglyceride, as well as a loss of normal secreted signals from adipocytes.

#### Gestational Diabetes

Glucose intolerance that is first identified during pregnancy is referred to as gestational diabetes. The importance of this classification is the significant maternal and fetal morbidity related to diabetes during pregnancy, warranting screening for diabetes mellitus in all pregnant women except those at low risk. In some cases gestational diabetes is merely the identification of glucose intolerance that was previously unrecognized until screening is performed during a pregnancy, or to the temporal development of diabetes (type 1 or type 2) during a pregnancy, perhaps accelerated by the pregnant state, but otherwise predestined to develop. In the majority of cases, however, glucose regulation returns to normal after the pregnancy. Pregnancy is characterized by progressively decreasing insulin sensitivity, due to the insulin antagonizing effects of increases in prolactin, human placental lactogen, estrogens, progesterone, and unbound cortisol levels. In the majority of pregnancies, this insulin resistance is compensated for by increased insulin secretion. However, in the 2–5% of pregnancies that develop gestational diabetes mellitus, there is an inadequate compensation. Thus, gestational diabetes represents the same or similar pathophysiologic abnormality of insulin sensitivity and insulin secretion that occurs in type 2 diabetes, with a portion of the insulin resistance being self-limited due to the pregnant state. However, there is also an underlying defect in insulin sensitivity and insulin secretion, and women who have had gestational diabetes are at a substantially increased risk for the future development of type 2 diabetes.

#### Metabolic Syndrome and Prediabetes

Insulin resistance often precedes the development of diabetes mellitus by many years. In addition, in some people, $\beta$-cell dysfunction does not develop, and insulin resistance exists indefinitely without progressing to diabetes. However, insulin resistance is not without significance of its own. The metabolic syndrome (also referred to as syndrome X) is an association of findings that frequently coexist, and includes insulin resistance, obesity (particularly abdominal/visceral rather than peripheral/subcutaneous), hypertension, and a specific form of dyslipidemia: hypertriglyceridemia and decreased high-density lipoprotein (HDL) cholesterol. These subjects are at risk of developing type 2 diabetes if they do not already have it, but more importantly, even in the absence of progression to diabetes, these individuals are at a markedly elevated risk for atherosclerotic vascular disease. The causality of obesity and insulin resistance in this syndrome is supported by the fact that lipid levels and hypertension may both be improved by decreasing insulin resistance through exercise and weight loss.

The diagnosis of diabetes mellitus is based on blood glucose levels that exceed defined levels, either in the fasting state or after a challenge with an ingested glucose load. The cutoffs are based mostly on levels that predict an increased risk for diabetic microvascular complications. Some individuals with insulin resistance will have glucose levels that do not meet criteria for the diagnosis of diabetes, but are above levels considered normal. Some of these subjects with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) will have glucose levels that revert to normal, or remain "impaired" over time. However, a large percentage will have progressive $\beta$-cell dysfunction (or worsening insulin resistance, or both) that results in progression to type 2 diabetes – ~40% in 5–10 years.

## Complications

### Acute

#### Ketoacidosis

Diabetic ketoacidosis (DKA) is the life-threatening acute metabolic decompensation in patients with diabetes mellitus. Because the development of DKA is suppressed by even modest insulin action, DKA is generally only a risk for patients with type 1 diabetes. The pathophysiology of DKA is one of acidosis, hyperosmolarity, and dehydration.

Decompensation towards DKA begins with a fall in insulin action, either due to an absolute decline in the insulin level, or due to antagonism of insulin action by cytokines and stress hormones, including catecholamines, cortisol, glucagons, and growth hormone. As insulin action becomes inadequate, serum glucose levels rise; the loss of insulin suppression of gluconeogenesis and glycogenolysis in the liver results in an increase in hepatic glucose output, and the loss of insulin stimulation of glucose uptake in muscle and adipose tissue results in accumulation of the excess glucose in the blood. The loss of insulin suppression results in unrestrained lipolysis in the adipocyte, releasing free

fatty acids and glycerol. Increased protein breakdown in muscle releases amino acids. These metabolites are taken up by the hepatocytes, where the free fatty acids are metabolized to ketoacids ($\beta$-hydroxybutyrate and acetoacetic acid), and the glycerol and amino acids serve as substrates for accelerated gluconeogenesis.

The hyperglycemia and ketoacidosis are initially moderated by the loss of glucose and ketoacids in the urine. However, the glucosuria produces an osmotic diuresis, and the ketoacidosis produce an ileus leading to nausea and vomiting that impairs the ability of the patient to drink to maintain their hydration. As dehydration develops, then worsens, the glomerular filtration rate of the kidney falls, and less glucose and ketoacids can be excreted in the urine, resulting in dramatic accumulation of glucose and ketoacids in the blood. The evolving disease stimulates (further) release of stress hormones that antagonize any remaining insulin action, and further exacerbate the hyperglycemia. The acidosis and the hyperosmolarity caused by the extreme hyperglycemia decrease cerebral function and impair the patient's consciousness, further compromising the patient's ability to drink. Without intervention, death is inevitable.

### Nonketotic Hyperosmolar, Coma

Patients with type 2 diabetes are not at much risk of developing DKA. They will generally have sufficient insulin action to restrain lipolysis, limiting free fatty acid delivery to the liver and subsequent ketoacid production. However, decompensation similar to that of DKA, but without the excess ketoacid production, can occur in patients with type 2 diabetes. Nonketotic hyperosmolar coma in patients with type 2 diabetes generally occurs when another illness induces a stress response, and there is an impaired ability to maintain hydration with oral fluids. As in DKA, the increase in stress hormones antagonize insulin action, resulting in increased hepatic glucose production and decreased peripheral glucose utilization. The rising hyperglycemia produces a glucosuria-driven osmotic diuresis. Dehydration is a result of an inability of oral fluid intake to compensate for the osmotic diuresis. As in DKA, the dehydration impairs the renal excretion of glucose, allowing the glucose level to continue to rise. The dehydration and hyperosmolarity impair the patient's consciousness, and oral intake falls further. The final situation is one of marked hyperglycemia, hyperosmolarity, and dehydration. As in DKA, the mortality of nonketotic hyperosmolar coma is high.

## Chronic

Patients with diabetes will have bothersome symptoms and are at risk of severe acute metabolic decompensation, even if treatment is successful in avoiding these issues, diabetes can lead to long-term complications that develop over years. These complications can be categorized as microvascular, which are unique to patients with diabetes, and macrovascular, which are complications of atherosclerotic vascular disease that also occur in people without diabetes, but occur with higher frequency in those with diabetes. These complications are felt to be due to a failure of treatment to perfectly normalize insulin and glucose levels, as well as to changes produced by insulin resistance in type 2 diabetes.

### Macrovascular Disease

As in individuals without diabetes, atherosclerotic changes in medium- and large-sized arteries that either partly or completely occlude the vessel lumen lead to morbidity and mortality due to impaired blood flow to the heart (angina and myocardial infarction), the brain (stroke), or the periphery (claudication and ischemic damage requiring amputation). These complications occur at a higher frequency and develop at younger ages in patients with diabetes; over half of patients with type 2 diabetes die from cardiovascular disease. It is not fully clear how diabetes contributes to the risk of atherosclerotic vascular disease. The factors that have been implicated include lipid abnormalities, hyperglycemia, and increases in prothrombotic and proinflammatory factors. In addition, the coexistence of other atherosclerotic risk factors, most notably hypertension in obese patients with type 2 diabetes, also plays a role.

In patients with type 2 diabetes, the insulin resistance that is a part of the pathophysiology is associated with a specific pattern of lipid abnormality that increases the atherosclerotic risk: decreased high density lipoprotein (HDL) cholesterol concentration, and elevated triglyceride concentration in the serum. In addition, the inadequate insulin action in poorly controlled diabetes of any type leads to inadequate stimulation of lipoprotein lipase activity and to increased lipolysis and elevated free fatty acids. The free fatty acids that are not oxidized to ketones in the liver are re-esterified to triglycerides and secreted as very low-density lipoproteins (VLDL). Thus, poorly controlled diabetes can lead to atherosclerotic risk by increasing triglyceride and VLDL levels; hypertriglyceridemia can exacerbate the risk by depressing HDL levels. Finally, hyperglycemia may contribute to disordered serum lipids by reducing expression of the heparin sulfate proteoglycan perlecan on hepatocytes, leading to increased levels of the atherogenic cholesterol-enriched apolipoprotein B-containing remnant particles.

Endothelial dysfunction is central to the development of atherosclerosis, with decreased production of the vasodilating molecule nitric oxide and increased production of the prothrombotic factor plasminogen

activator inhibitor-1 (PAI-1) two factors implicated in this dysfunction. In diabetes, hyperglycemia inhibits nitric oxide production and increases production of PAI-1. Hyperglycemia may also contribute to macrovascular disease indirectly through microvascular damage to the nutrient blood vessels supplying the walls of the medium- and large-sized arteries.

Another aspect that is likely to play a role in the development of atherosclerosis in type 2 diabetes relates to the pathway-specific insulin resistance that is present. As noted previously, the insulin resistance of type 2 diabetes has less of an effect on the activation of the intracellular mitogen-activated protein (MAP) kinase pathway than the pathway activating phosphatidylinositol-3-kinase. This may lead to increased activation of the MAP kinase pathway by insulin signaling, which also decreases nitric oxide production and increases PAI-1 production. In addition, as the MAP kinase pathway is a mitogenic pathway, the increased MAP kinase signaling can increase proliferation of the vascular smooth muscle cells, contributing to the atherosclerotic process.

**Microvascular Disease**

The hyperglycemia of diabetes mellitus leads to damage to the microvascular circulation that results in tissue and organ damage, most notably in the retina, kidneys, and nerves. Due to these microvascular complications, diabetes mellitus is a leading cause of blindness, end-stage renal disease, and neuropathy. Increasing degrees of hyperglycemia are the main risk factor for microvascular complications, with the Diabetes Control and Prevention Trial (for type 1 diabetes) and the United Kingdom Prospective Diabetes Study (for type 2) definitively demonstrating a decreased incidence of microvascular complications with treatments that lower the degree of hyperglycemia. Unfortunately, even the best of current therapies do not completely normalize blood glucose levels. The risk of microvascular complications increases with time, with the earliest evidence uncommon prior to a duration of hyperglycemia of 5 years; note, however, that except for typical type 1 diabetes, hyperglycemia may be present for years before the diagnosis of diabetes is made. Finally, as yet undefined genetic factors also play a role in the risk of microvascular disease.

Microvascular disease begins with abnormal blood flow and increased vascular permeability due to decreased activity of vasodilators, increased activity of vasoconstrictors, and overexpression of permeability factors. Irreversible increases in vascular permeability occur with alterations in the extracellular matrix. Ultimately, there is cell loss, and capillary occlusion from deposition of extravasated plasma proteins and the overproduction of extracellular matrix. Microvascular disease affects the glomerulus in the kidney, with the earliest evidence being leakage of albumin into the urine and progressing to loss of glomerular function. Damage to retinal capillaries causes hypoxic-ischemia, edema, new vessel formation, and hemorrhage. It is the development of new vessels, referred to as proliferative retinopathy, that leads to retinal detachment, vitreous hemorrhage, and loss of sight. Diabetic neuropathy includes degeneration of motor, sensory, and autonomic nerves.

While it is clear that hyperglycemia leads to microvascular disease, the precise mechanism for this has not been completely demonstrated. However, four pathologic processes triggered by hyperglycemia have been identified and proposed as causative mechanisms: increases in the polyol and hexosamine pathways, production of advanced glycation end-products (AGEs), and activation of protein kinase C (PKC). *In vivo* studies with inhibitors, either in animal models or in human subjects, have supported a role for each of these pathways (except the recently implicated hexosamine pathway) in microvascular pathology. The relative contribution of each of these proposed mechanisms likely varies across cell types.

While mechanisms exist for hyperglycemia to directly activate each of the four potential microvascular pathogenic pathways, they may also be activated by the hyperglycemia-induced overproduction of superoxide. The increased metabolism of glucose in the presence of hyperglycemia increases the delivery of electron donors from the tricarboxylic acid (TCA) cycle to mitochondria. This can lead to an increase of the proton gradient across the inner mitochondrial membrane, inhibiting the electron transport chain and increasing superoxide production. Because superoxide inhibits the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), precursors (as noted) can accumulate and be diverted into the polyol pathway (glucose), the hexosamine pathway (fructose-6-phosphate), production of DAG (glyceraldehyde-3-phosphate) which activates PKC isoforms, and production of the reactive dicarbonyl methylglyoxal (glyceraldehyde-3-phosphate) which produces AGEs.

***Polyol Pathway*** Hyperglycemia drives an increased intracellular generation of sorbitol through the NADPH-dependent reduction of glucose by aldose reductase. Increased production of sorbitol could alter cellular functions in a number of ways, including increasing intracellular osmotic stress, decreasing myoinositol and taurine (as compensation for the increase in sorbitol in order to prevent or mitigate an increase in osmolarity), or through an increased NADH/$NAD^+$ ratio (due to $NAD^+$-dependent oxidation of sorbitol to fructose). However, the most likely mechanism for cellular dysfunction caused by increased flux through the polyol pathway is due to the consumption of NADPH.

This leads to a lack of NADPH available to regenerate reduced glutathione, limiting the ability of the cell to protect itself against oxidative stress.

*Hexosamine Pathway* The hexosamine pathway generates UDP-*N*-acetylglucosamine (GlcNAc) from glucose to provide substrate for proteoglycan synthesis and for O-linked glycosylation of certain proteins. The activity or function of many proteins, including transcription factors, can be modified by O-linked glycosylation, often in concert with reciprocal modification by phosphorylation. With hyperglycemia, some of the excess glucose is shunted into the hexosamine pathway. As noted previously, increased flux through this pathway may play a role in insulin resistance. In addition, increased generation of UDP-GlcNAc could alter gene expression and cellular function through excess protein glycosylation, contributing to the pathogenesis of microvascular disease.

*Advanced Glycation End-Products* Glucose will react with free amino groups on proteins in a nonenzymatic reaction to form glycated proteins. This reaction starts with the formation of a Schiff base (aldamine), which then undergoes an internal Amadori rearrangement. The formation of the Schiff base is rapid and reversible, while the Amadori rearrangement is much slower and results in a more stable ketoamine. This process occurs at a rate proportional to the prevailing glucose level that a given protein is exposed to. The glycation of hemoglobin is used clinically to estimate the average blood sugar in patients with diabetes, as the percent of hemoglobin in the glycated $A_{1C}$ form ($HbA_{1C}$) is proportional to the average glucose level present over ~3 months prior to testing (roughly the red blood cell life span).

Proteins modified irreversibly through nonenzymatic reaction with glucose or glucose-derived compounds are referred to as advanced glycation end-products (AGEs). Most AGEs are not formed through the reaction of proteins directly with glucose but rather with three reactive dicarbonyl compounds that are produced intracellularly from glucose: glyoxal (an auto-oxidation product of glucose), 3-deoxyglucasone (formed from decomposition of the Amadori product of glucose), and methylglyoxal (from fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate generated from glycolysis). The reactive dicarbonyls interact with the amino groups of proteins, and can introduce both intra- and intermolecular cross-links. AGE formation may lead to microvascular damage through alteration of the function of both intracellular and extracellular proteins modified in this way. In particular, the modification of extracellular matrix proteins by this process is likely to contribute to abnormal vascular permeability and elasticity. In addition, however, AGEs formed from plasma proteins bind to and activate specific AGE receptors on endothelial cells, mesangial cells, and macrophages, (one such receptor is RAGE, a member of the immunoglobin superfamily). Activation of AGE receptors induces production of cytokines, growth factors, procoagulatory factors, and reactive oxygen species. Induction of vascular endothelial growth factor (VEGF) by AGE receptor activation most likely contributes to the increased permeability of capillary walls present in diabetes.

*Activation of PKC* Hyperglycemia increases intracellular diacylglycerol (DAG) levels, which is an activator of many of the PKC isoforms. Hyperglycemia may also activate PKC isoforms indirectly by increasing reactive oxygen species from activation of AGE receptors and from increased activity of the polyol pathway. As discussed above, activation of PKC may play a role in hyperglycemia-induced insulin resistance. Mechanisms through which activation of PKC may be involved in the development of microvascular disease include: inhibiting production of the vasodilator nitric oxide; increasing activity of the vasoconstrictor endothelin-1; increasing expression of VEGF, leading to increased cell permeability; and contributing to the accumulation of extracellular matrix protein by inducing expression of TGF-$\beta$1, fibronectin, and type IV collagen.

## Diabetes Treatment Medications – Mechanisms of Action

Patients with diabetes of any type can be treated with insulin to overcome the relative or absolute insulin deficiency. For patients with type 1 diabetes, the loss of the ability to produce endogenous insulin because of the loss of pancreatic $\beta$-cells makes insulin the only treatment option. Similarly, in many patients with type 2 diabetes, the $\beta$-cell dysfunction will progress to a degree that insulin treatment is necessary to maintain glycemic control. However, for many patients with type 2 diabetes, a number of treatments may be effective in lowering the degree of hyperglycemia.

### Weight Loss and Exercise

For the majority of patients with type 2 diabetes, obesity is a significant contributor to the insulin resistance that underlies their disease. Therefore, in many cases, if the patient is able to lose weight, insulin sensitivity will improve sufficiently to return metabolic control to normal or near normal. In many cases, only modest weight loss can be effective. A sedentary lifestyle also leads to decreased insulin sensitivity. Therefore, exercise

to improve fitness can also improve insulin sensitivity sufficient to ameliorate the diabetic state, an effect that can occur in the absence of weight loss. AMP-activated kinase (AMPK) functions as an intracellular energy sensor. During exercise, activation of AMPK increases glucose uptake into muscle by stimulating GLUT4 translocation to the plasma membrane in a noninsulin receptor dependent pathway. In addition, the exercise-stimulated activation of AMPK may lead to a prolonged increase in insulin sensitivity by increasing expression of GLUT4, and increasing the insulin sensitivity of the liver.

## Insulin Secretagogues

Sulfonylureas and the newer meglitinide analogues lower blood sugar levels by stimulating endogenous insulin secretion. They act by binding to the sulfonylurea receptor (SUR) – one component of the $\beta$-cell potassium channel, the other being $Kir_{6.2}$. Binding of these agents to SUR results in closure of the potassium channel, mimicking the effect of a glucose-driven increase in the cellular ATP/ADP ratio, and setting in motion the intracellular signal for exocytosis of insulin. Unfortunately, due to the worsening $\beta$-cell dysfunction in most patients with type 2 diabetes, insulin secretagogues often become ineffective over time.

## Metformin

Metformin is a biguanide compound that works by increasing insulin sensitivity in diabetic patients. Its main effect is on the liver, to decrease excess hepatic glucose production. The underlying mechanism for metformin's action has not been fully determined. However, it appears to function by activating AMPK. By activating AMPK, the hepatocyte shifts from lipogenesis to fatty-acid oxidation. This reduces hepatic triglyceride content, which has been implicated in the cause of insulin resistance. How metformin activates AMP-kinase is uncertain, but may involve inhibition of the mitochondrial electron transport chain.

## Thiazolidinediones

Thiazolidinediones (TZDs) are a second class of insulin-sensitizing drugs. In contrast to metformin, however, their primary action is to increase peripheral insulin sensitivity, increasing glucose uptake into fat and muscle. TZDs are ligands for the peroxisome proliferator-activated receptor-$\gamma$ (PPAR-$\gamma$), which is expressed in high levels only in adipose tissue, although low levels of expression are seen in other tissues, including in muscle, liver, and pancreatic $\beta$-cells. PPAR-$\gamma$ is a transcription factor that alters gene expression in a ligand-dependent manner. The importance of PPAR-$\gamma$ in the pathophysiology of diabetes is highlighted by the fact that individuals homozygous for a common amino acid polymorphism (Pro12Ala) in the *PPAR*$\gamma$ gene are more insulin resistant and have an increased risk of diabetes than individuals with one Ala12 allele. The ability of PPAR-$\gamma$ to improve overall carbohydrate control in spite of the fact that adipocytes are responsible for a relatively minor fraction of insulin-stimulated glucose uptake compared to muscle may be due to direct effects of TZDs on muscle. However, it is most likely that TZDs exert their effect by altering gene expression in adipocytes, which leads to improved insulin action in muscle and liver.

## $\alpha$-Glucosidase Inhibitors

Acarbose and miglitol inhibit the intestinal enzyme $\alpha$-glucosidase. Given with meals, this delays carbohydrate absorption, leading to a requirement for a lower maximal insulin level to dispose of the absorbed carbohydrate. These drugs do not significantly affect fasting glucose levels, and so are generally ineffective as single agent therapy. However, there is accumulating information demonstrating the importance of controlling postprandial hyperglycemia to minimize long-term diabetic complications.

## See Also the Following Articles

Glycogen Metabolism • Insulin- and Glucagon-Secreting Cells of the Pancreas • Insulin Receptor Family • Phosphatidylinositol-3-Phosphate • Protein Kinase C Family

## Glossary

**$\beta$-cell** The insulin producing cells in the Islets of Langerhans.

**glucose disposal** The removal of glucose from the blood, usually in the context of its removal after having been added, as after absorption of a carbohydrate-containing meal.

**glucose intolerance** The condition where the concentration of glucose in the blood after ingestion of carbohydrate is elevated above normal; may also refer more generally to a condition associated with hyperglycemia.

**hyperglycemia** An abnormally elevated concentration of glucose in the blood.

**kinase** An enzyme that modifies a substrate through the addition of a phosphate group.

**osmotic diuresis** An increase in urine output stimulated by an increase in osmotically active solute in the urine; in diabetes, the solute is glucose.

**phosphatase** An enzyme that modifies a substrate through the removal of a phosphate group.

**prandial** Meal associated.

## Further Reading

Bell, G. I., and Polonsky, K. S. (2001). Diabetes mellitus and genetically programmed defects in $\beta$-cell function. *Nature* **414**, 788–791.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813–820.

Cefalu, W. T. (2001). Insulin resistance: cellular and clinical concepts. *Exp. Biol. Med.* **226**, 13–26.

DeFronzo, R. A. (1997). Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes genes. *Diabetes Rev.* **5**, 177–269.

Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2002). Oxidative stress and stess-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endo. Rev.* **23**, 599–622.

Kahn, S. E. (2003). The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysioloy of Type 2 diabetes. *Diabetologia* **46**, 3–19.

Maechler, P., and Wollheim, C. B. (2001). Mitochondrial function in normal and diabetic $\beta$-cells. *Nature* **414**, 807–812.

Mathis, D., Vence, L., and Benoist, C. (2001). $\beta$-Cell death during progression to diabetes. *Nature* **414**, 792–798.

Moller, D. E. (2001). New drug targets for type 2 diabetes and the metabolic sydrome. *Nature* **414**, 821–827.

Saltiel, A. L., and Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799–806.

Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. *J. Clin. Invest.* **106**, 171–176.

The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2003). The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* **26** (suppl. 1), S5–S20.

Zimmet, P., Alberti, K. G. M. M., and Shaw, J. (2001). Global and societal implications of the diabetes epidemic. *Nature* **414**, 782–787.

## BIOGRAPHY

David W. Cooke is an Associate Professor of Pediatrics in the Division of Pediatric Endocrinology at the Johns Hopkins University School of Medicine. His research interest is the molecular basis of insulin resistance, particularly that occurring in the adipocyte. He received S.B. degrees in Mechanical Engineering and Biology from the Massachusetts Institute of Technology and his M.D. from the Duke University School of Medicine. He received his pediatric residency training and his pediatric endocrine fellowship training at the Johns Hopkins Hospital.

# Diacylglycerol Kinases and Phosphatidic Acid Phosphatases

**Stephen M. Prescott and Matthew K. Topham**
*Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah, USA*

**Diacylglycerol (DAG) is an important lipid that initiates specific intracellular signaling events. The majority of signaling DAG is generated by hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) by the enzyme phospholipase C (PLC). DAG activates protein kinase C (PKC) isoforms, binds to and activates the RasGRP nucleotide exchange factors, and recruits the chimaerins, which are Rac GTPase activating proteins (GAPs), to membrane compartments. Because DAG can modulate a plethora of signaling events, it is crucial that intracellular DAG levels be tightly regulated. Though DAG can be metabolized in various ways, under most circumstances its major route for metabolism is by its phosphorylation (Figure 1), a reaction that is catalyzed by the diacylglycerol kinases (DGKs).**

## The Diacylglycerol Kinases

Nine mammalian DGK isoforms have been identified (Figure 2). The heterogeneity of the gene family is similar to the PKC and PLC families, suggesting that the DGKs are not simply lipid biosynthetic enzymes, but that they also have signaling roles, since enzymes involved in biosynthetic pathways usually do not have extended families. One or only a few DGK isoforms have been identified in organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arabidopsis thaliana*, and no DGK gene has been identified in yeast. The structural diversity of DGKs in mammals suggests that they may have roles in processes specific to higher vertebrates.

DGK isoforms are categorized based on shared structural motifs. All DGK isoforms have a catalytic domain that is necessary for kinase activity. In most cases, the catalytic domain is a single motif, but DGKs $\delta$ and $\eta$ have bipartite catalytic domains. All DGKs have at least two cysteine-rich regions homologous to the C1A and C1B motifs of PKCs. In theory, these domains may bind DAG, perhaps localizing DGKs to where DAG accumulates. However, no DGK C1 domain has so far been conclusively shown to bind DAG. Structural predictions suggest that most DGK C1 domains may not bind DAG, and most DGKs tested are unable to bind long-lived DAG-like analogues. Houssa and van Blitterswijk noted that in DGKs, the C1 domain closest to the catalytic domain is highly conserved, including an extended motif of 15 amino acids not present in other C1 domains. Conserved residues in this extended motif have been shown to be critical for DAG kinase activity. Together, these data suggest that DGK C1 domains are structurally different from C1 domains in other proteins and that the different C1 domains of a single DGK isoform may have unique functions. Distinct functions of individual C1 domains remain to be definitively demonstrated.

In addition to the C1 and catalytic domains, other regulatory domains are used to group the DGKs into five subfamilies. Type I DGKs have calcium-binding EF hand motifs and have been shown to be more active in the presence of calcium. Type II DGKs have a pleckstrin homology (PH) domain at their amino termini. This domain in DGK$\delta$ has been shown to bind weakly and nonselectively to phosphatidylinositols (PIs). DGK$\delta$ also has a sterile alpha motif (SAM domain) at its C-terminus that helps localize it to the endoplasmic reticulum (ER). DGK$\varepsilon$, a type III enzyme, has an unusual specificity toward acyl chains of DAG, strongly preferring a specific fatty acid – arachidonate – at the *sn*-2 position. This preference suggests that DGK$\varepsilon$ may be a component of the biochemical pathway that accounts for the enrichment of phosphatidylinositols with arachidonate. Type IV DGKs have a MARCKS phosphorylation domain and, at their C-termini, four ankyrin repeats. The type V enzyme, DGK$\theta$, has three C1 domains and a PH domain.

## Regulation of DGK Activity

Activation of the DGKs is complex and unique for each DGK isotype. In most cases, DGKs must translocate to a membrane compartment to access DAG. In addition, their activity can be modified by appropriate cofactors, and several DAG kinases are also regulated by

 © 2004, Elsevier Inc. All Rights Reserved. 

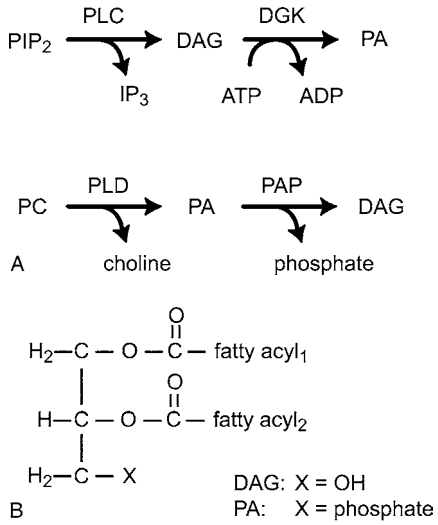



**FIGURE 1** (A) Different enzymatic pathways can produce diacylglycerol (DAG) and phosphatidic acid (PA). Phosphatidylinositol-specific PLC enzymes generate DAG that can be phosphorylated by DGKs to produce PA. In another pathway, phospholipase D (PLD) hydrolyzes phosphatidylcholine (PC) – or phosphatidylethanolamine – making PA that can be further hydrolyzed by PAPs to generate DAG. (B) DAG and PA have the same general structure but contain different functional groups attached to the third (*sn*-3) carbon. Depending on their molecular structure, the fatty acyl groups confer different signaling properties to the DAG or PA.

post-translational modifications. Finally, tissue-specific alternative splicing of DGKs $\beta$, $\delta$, and $\zeta$, and probably other isotypes, allows additional opportunities for regulation. This complexity permits tissue- or cell-specific regulation of each DGK isotype, depending on the availability of cofactors and the type of stimulus that the cell receives.

DGK$\alpha$ is an example of the contextually dependent differential regulation of DGKs. DGK$\alpha$ translocates to at least two different membrane compartments in T lymphocytes depending upon the agonist used to activate the cells: from the cytosol to a perinuclear region in T cells stimulated with IL-2, and to the plasma membrane upon activation of the T cell antigen receptor. Once at a membrane compartment, the DAG kinase activity of DGK$\alpha$ can be modified by the availability of several cofactors. Calcium is known to bind to EF hand structures and stimulates DGK$\alpha$ activity *in vitro*. Lipids also modify its activity: phosphatidylserine and sphingosine activate DGK$\alpha$ *in vitro* and likely *in vivo* as well. Finally, DGK$\alpha$ can be phosphorylated by several protein kinases including some PKC isoforms and Src kinase, which may enhance its DAG kinase activity.

Like DGK$\alpha$, other DGK isotypes appear to be sensitively regulated. For example, type II DGKs have a PH domain that may help localize these DGKs, modify their activity, or allow binding to other proteins. The PH domain of the type II DGK$\delta$ binds phosphatidylinositols but its DAG kinase activity is not affected by these lipids. In contrast, the activity of DGK types III and IV can be modified by phosphatidylinositols and by phosphatidylserine, sometimes in opposing ways. For example, the type III enzyme, DGK$\varepsilon$, is inhibited by, whereas type IV DGKs are activated by, phosphatidylserine. Type IV DGKs are strongly regulated by subcellular translocation. These enzymes have a nuclear localization signal that is regulated by PKC phosphorylation, and there is evidence that the syntrophin family of scaffolding proteins further regulates their subcellular location by anchoring them in the cytoplasm. Finally, DGK$\theta$, a type V DGK, can be regulated through its association with active RhoA, which abolishes its DAG kinase activity and is the only known example of direct regulation of a DGK activity through a protein–protein interaction. Thus, depending on the context of activation, the availability of cofactors, and the activation state of protein kinases, DGKs can be differentially regulated.

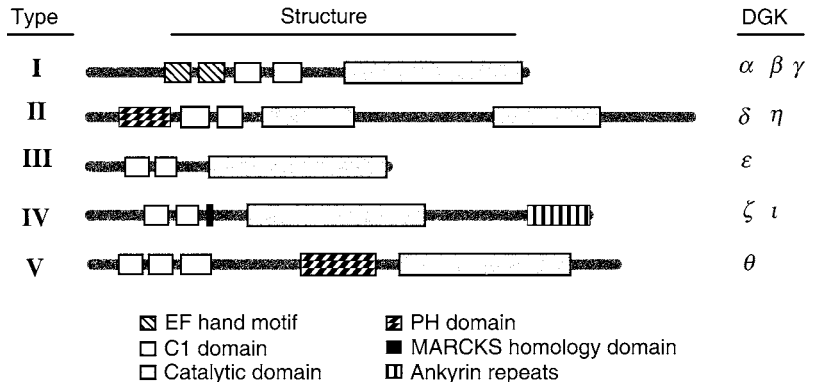



**FIGURE 2** The mammalian DGK family. Based on structural motifs, the nine mammalian DGKs are divided into five subtypes. Alternative splicing of some DGK isotypes generates even more structural diversity. Many of the DGKs contain other unique structural domains of unclear significance that are not shown.

## Activity of DGKs is Compartmentalized

Evidence suggests that DGK activity is restricted to the area of localized DAG pools generated after activation of receptors. This concept was initially tested in 1994 by Van der Bend *et al.*, who detected DGK activity in cells following receptor activation but could not detect significant DAG kinase activity after treating the cells with exogenous PLC, which caused global, nonspecific DAG generation. Their data suggest that DGKs are active only in spatially restricted compartments following physiologic generation of DAG.

We are only beginning to understand the complexities of spatially restricted DGK function. Several DGKs localize to the cytoskeleton, where they may participate in regulating cytoskeletal dynamics. Investigators have noted that DGK activity associates with a complex of proteins including PI5K, Rac, Rho, Cdc42, and Rho-GDI, all of which regulate cytoskeleton dynamics. Houssa *et al.* showed that RhoA associates with DGKθ – but only when RhoA is in its active form – and that this interaction abolished DGK activity. RhoA appears to bind the catalytic domain of DGKθ, which likely leads to the disruption of DAG kinase activity. DGK$\zeta$ localizes at the leading edge and cell extensions of glioblastoma cells and can disrupt cell spreading when overexpressed. It and several other DGK isotypes co-immunoprecipitate with Rho family proteins when overexpressed in cells. The physiologic significance of these interactions is not clear, though together, these data suggest that DGKs likely have a role in regulating the cytoskeleton.

In the nucleus, a distinct nuclear phosphatidylinositol cycle is regulated separately from its plasma membrane/cytosolic counterpart. The function of DAG produced in the nucleus has received little attention, but data clearly indicate that in most cases it acts to promote cell growth independently of plasma membrane DAG. For example, some growth factors (e.g., IGF-1) can stimulate a pulse of nuclear DAG without causing a measurable change in extranuclear DAG, and several groups have demonstrated that nuclear DAG fluctuates independently of extranuclear DAG during the cell cycle. Nuclear DAG was shown to peak shortly before S phase, suggesting that it may participate in the G1/S transition. Supporting this conclusion and emphasizing the importance of DAG signaling in the cell cycle, we demonstrated that when a nuclear DGK was overexpressed, cells accumulated at the G0/G1 transition, presumably because the kinase attenuated the nuclear DAG concentration. Within the nucleus, DAG signaling, like its extranuclear counterpart, appears to be compartmentalized. For example, in 1999 D'Stantos *et al.* demonstrated independently fluctuating pools of nuclear diacylglycerol with distinct fatty acid compositions, strongly suggesting the existence of multiple, differentially regulated pools of nuclear DAG.

DGKs are present in nuclei and appear to have prominent and specific roles there. DGKs $\alpha$, $\zeta$, and ι, translocate to the nucleus, while a fraction of DGKθ is localized there constitutively. Different DGK subtypes are confined to specific, separate compartments within the nucleus, suggesting specificity in their nuclear roles. For example, DGK$\alpha$ associates with the nuclear envelope, while DGKθ and DGK$\zeta$ are found in discrete regions within the body of the nucleus. Evidence suggests that DGKs likely affect nuclear signaling either by terminating DAG signals or by generating PA. For example, nuclear DGK$\zeta$ inhibits progression from G1 to S phase of the cell cycle, likely by metabolizing DAG. Conversely, in T lymphocytes, PA generated by nuclear DGK$\alpha$ appears to be necessary for IL-2-mediated progression to S phase of the cell cycle. Thus, DGK$\alpha$ and DGK$\zeta$ appear to have opposing roles in the nucleus, reflecting the complexity of lipid signaling and DGK activity there.

## Coupling of DGKs with Other Signaling Proteins

Recent evidence suggests that in addition to acting locally at sites of DAG pools, DGKs can specifically associate with and regulate signaling proteins that are activated by either DAG or PA. For example, DGK$\zeta$ associates with and inhibits RasGRP1, a guanine nucleotide-exchange factor for Ras that requires DAG to function. This regulation may be selective: five other DGK isotypes did not significantly inhibit RasGRP1 activity. In *Caenorhabditis elegans*, Nurrish *et al.* found that *dgk-1*, an ortholog of human DGKθ, regulates DAG signaling that is necessary for acetylcholine release. Combined, these data demonstrate that DGK activity is not only targeted at sites of DAG production, but is also specifically directed toward a subset of DAG-activated proteins.

## PA as a Signal

The DGK reaction is of special interest because it removes one signaling molecule, DAG, while generating another, phosphatidic acid (PA). PA can stimulate DNA synthesis and is potentially mitogenic. It modulates the activity of several enzymes, including phosphatidylinositol 5-kinases (PI5Ks), PAK1, Ras-GAP, and PKC$\zeta$, and it has a prominent role in vesicle trafficking. At the plasma membrane, PA also helps recruit Raf to the Ras signaling complex. Although phospholipase D (PLD)

generates the bulk of signaling PA (Figure 1), DGKs likely also contribute to its intracellular concentration.

The PA species generated by DGK and PLD reactions are distinct from each other by virtue of their initial substrates. Phosphatidylcholine, which is largely composed of saturated and mono-unsaturated fatty acids is the predominant substrate of PLD, while PA produced by DGKs is derived from DAG that is enriched in polyunsaturated fatty acids – particularly arachidonate. There is good evidence that each PA species – saturated and unsaturated – can differentially activate targets. For example, saturated PA species induce MAPK activation to a greater extent than unsaturated PAs; Flores and colleagues presented evidence that the PA produced by DGK$\alpha$ was necessary for stimulated T lymphocytes to progress to S phase of the cell cycle. Thus, DGKs can influence signaling events either by metabolizing DAG or by generating PA.

## PA Conversion to DAG by PA Phosphatase

Just as PA can be generated by more than one enzymatic pathway, DAG is also the product of several enzymatic reactions. The PLC reaction is generally thought to be the major route to generate signaling DAG, but some lipid phosphate phosphatases (LPPs) can also produce DAG by dephosphorylating phosphatidic acid (Figure 1). Most LPPs are integral membrane proteins thought to act on extracellular lipids, but some of them are clearly intracellular proteins. Type I LPPs specifically dephosphorylate phosphatidic acid and are also known as PA phosphatases (PAPs). Though their cDNAs have not been cloned and the proteins have not been purified to homogeneity, PAPs are known to be cytosolic proteins that can translocate to membrane compartments. Historically, PAP activity is thought to be coupled with PLD activity to generate DAG. But very little published work supports that this DAG can initiate signaling. Due to the identity of its precursor, DAG generated by PAP activity is composed of predominantly saturated fatty acids, and like PA, the signaling properties of DAG depend on these fatty acid components. Evidence suggests that DAG composed of saturated fatty acids – like that produced by PAP activity – has weak signaling properties compared to unsaturated DAG. Thus, PAPs are not thought to initiate DAG signaling. Instead, PAPs may terminate PA signals that were initiated by phospholipase D. It is possible that PAP activity could couple with DGK activity to regenerate unsaturated DAG that could initiate signaling, but no one has demonstrated that this cycle exists. Thus, PAPs theoretically could contribute to DAG signaling but evidence is lacking that they do so; it is generally agreed that PAP activity is coupled with PLDs and not DGKs.

## Conclusions

As evidence accumulates, it is apparent that the structural diversity of DGK isoforms mirrors the range of functions and sites of action of these enzymes. Further studies of the regulation of lipid signaling should reveal additional roles for DGKs and more precise information about their regulation. Phosphatidic acid phosphatases, in contrast, appear to be a less diverse family of enzymes that are coupled to PLD activity.

### See Also the Following Articles

Phosphatidylinositol Bisphosphate and Trisphosphate • Phospholipase C

### Glossary

**diacylglycerol** A lipid composed of a glycerol backbone with fatty acids attached by ester bonds to its first (*sn-1*) and second (*sn-2*) carbons.

**fatty acid** Monobasic acids containing long hydrocarbon chains that can be either saturated (no double bonds) or unsaturated (one or more double bonds).

**phosphatidic acid** Diacylglycerol with a phosphate group attached to the third (*sn-3*) carbon of the glycerol backbone.

**phosphatidylinositol** Phosphatidic acid with an inositol group attached to the phosphate.

**phospholipid** The major structural lipids of most cellular membranes. They contain one or more phosphate groups and, if derived from glycerol, are known as phosphoglycerides.

### Further Reading

Hodgkin, M. N., Pettitt, T. R., Martin, A., Michell, R. H., Pemberton, A. J., and Wakelam, M. J. O. (1998). Diacylglycerols and phosphatidates: Which molecular species are intracellular messengers? *Trends Biochem. Sci.* **23**, 200–204.

Nurrish, S., Segalat, L., and Kaplan, J. M. (1999). Serotonin inhibition of synaptic transmission: G$\alpha_o$ decreases the abundance of UNC-13 at release sites. *Neuron* **24**, 231–242.

Sanjuan, M. A., Jones, D. R., Izquierdo, M., and Merida, I. (2001). Role of diacylglycerol kinase $\alpha$ in the attenuation of receptor signaling. *J. Cell Biol.* **153**, 207–219.

Sciorra, V. A., and Morris, A. J. (2002). Roles for lipid phosphate phosphatases in regulation of cellular signaling. *Biochim. Biophys. Acta* **1582**, 45–51.

Topham, M. K., and Prescott, S. M. (2001). Diacylglycerol kinase $\zeta$ regulates Ras activation by a novel mechanism. *J. Cell. Biol.* **152**, 1135–1143.

Topham, M. K., and Prescott, S. M. (2002). Diacylglycerol kinases: Regulation and signaling roles. *Thromb. Haemostasis* **88**, 912–918.

van Blitterswijk, W. J., and Houssa, B. (2000). Properties and functions of diacylglycerol kinases. *Cell. Signalling* **12**, 595–605.

Van der Bend, R. L., de Widt, J., Hilkmann, H., and van Blitterswijk, W. J. (1994). Diacylglycerol kinase in receptor-stimulated cells converts its substrate in a topologically restricted manner. *J. Biol. Chem.* **269**, 4098–4102.

## Biography

Stephen M. Prescott is the Executive Director of the Huntsman Cancer Institute at the University of Utah in Salt Lake City. His research explores the enzymes that generate lipid signals for growth and differentiation, and the role that they play in cancer. Another major area of interest is how inflammation is regulated.

Matthew K. Topham is an Assistant Professor of Internal Medicine at the University of Utah and an Investigator at the Huntsman Cancer Institute. He studies lipid signaling in cancer and is particularly interested in the diacylglycerol kinases and cyclooxygenases.

# Disulfide Bond Formation

**Hiram F. Gilbert**
*Baylor College of Medicine, Houston, Texas, USA*

**Disulfide bond formation is a posttranslational protein modification that introduces a covalent cross-link between the sulfhydryl groups of specific cysteine residues. Biologically, disulfide bonds are most often used to increase the conformational stability of extracellular proteins, but disulfides may also serve catalytic or regulatory roles through their effects on protein structure. Disulfide bonds are formed as the protein folds into its correct biological structure. Elaborate quality control systems in the bacterial periplasm and eukaryotic endoplasmic reticulum assure that correct disulfides are formed as the proper conformation is achieved.**

## Chemistry of Disulfide Formation as a Posttranslational Modification

### Disulfide Formation

Extracellular and secreted proteins often contain many disulfides, with pairs of cysteines linked to each other in a specific configuration in the folded protein (Figure 1). Biochemically, disulfide formation is an oxidation (the loss of electrons). Not all of the cysteines in a protein form disulfides. Disulfides form only when the structure of the protein places two cysteines in the proper spatial location and when an oxidizing agent is available. The chemical reaction is reversible, and high concentrations of a reductant can reverse or prevent disulfide formation.

### Disulfide Rearrangements

Through a chemical process called thiol-disulfide exchange, one disulfide may serve as an oxidizing agent to form a disulfide between two different cysteines (Figure 2). If this leads to connecting the cysteines in a different configuration, it is termed disulfide isomerization.

## Oxidative Protein Folding

Since many disulfides will be buried in the core of the protein and link cysteines that are not adjacent in sequence, disulfide formation and rearrangements often occur as the protein attains a three-dimensional structure (protein folding). The coupling of disulfide formation and protein folding is termed "oxidative protein folding."

### Structure is Linked to Disulfide Formation

In the 1960s, Anfinsen and his colleagues used the reversible nature of disulfide formation to demonstrate that the primary sequence of a protein contains sufficient information to define the final, biologically active structure of a protein. Disulfide bonds stabilize protein structure by organizing and destabilizing the denatured protein relative to the native structure. Usually, both the disulfides and noncovalent interactions (hydrophobic interaction, hydrogen bonds, etc.) are needed to specify the structure of ribonuclease A (RNase). Reducing the disulfides or using urea to disrupt the noncovalent structure causes a loss in biological activity. After removing the urea and including an oxidizing agent to reform the disulfides, the protein spontaneously refolds, forming the correct disulfides and a biologically active structure. However, if disulfide formation is permitted without removing the urea, random disulfides form (scrambled RNase) and there is no regain of biological activity. If disulfide formation is inhibited (by including a reducing agent), the native structure does not form when the urea is removed. Disulfide bonds are essential for forming the native structure but when the noncovalent structure is disrupted by denaturants disulfides are usually not sufficient to specify the tertiary structure.

### Disulfide Bond Formation during Protein Folding

Even with two disulfides, there are numerous ways to connect the cysteines (Figure 3), and the problem increases greatly as the number of disulfides to be formed increases. Early in protein folding, disulfide formation tends to be error-prone. Two types of

 © 2004, Elsevier Inc. All Rights Reserved. 

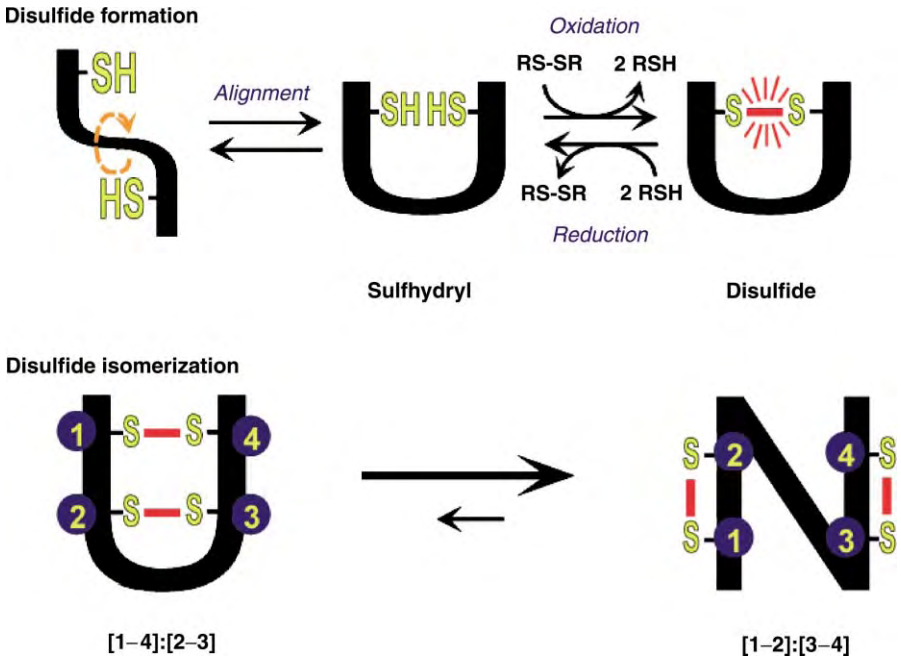



**FIGURE 1** Disulfide formation and isomerization. An alignment step is needed to bring two sulfhydryl groups from cysteine residues into close proximity. Disulfide formation represents an oxidation where the electrons from disulfide formation must be transferred to an oxidizing agent. Disulfide isomerization does not formally require a change in the oxidation state of the protein; however, a thiol group is needed to initiate and propagate the change in disulfide connectivity.

error have been found: disulfides may be formed between the wrong pairs of cysteines or the correct cysteines may be paired but in a temporal order that interferes with the assembly of the rest of the structure. In both cases, the incorrect disulfides are replaced with correct ones through disulfide isomerization. In the cases examined so far, many incorrect disulfides accumulate early in oxidative folding. These are slowly replaced by correct disulfides through isomerization which requires the breaking and reforming of disulfide bonds.

## Catalysis of Disulfide Formation

Soon after discovering that native proteins can be regenerated from reduced, denatured ones, Anfinsen realized that *in vitro* oxidative folding was often too

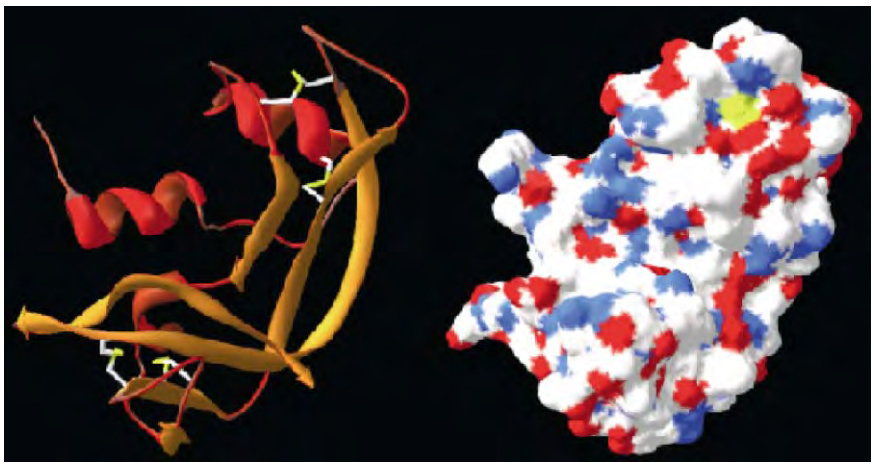


**FIGURE 2** Structure of disulfide bonds. (Left) Disulfide bonds cross-link cysteines in various parts of the protein structure. (Right) Some disulfides (yellow) are exposed to solution in the tertiary structure of the protein but others are buried in the core of the protein and not exposed to solution.

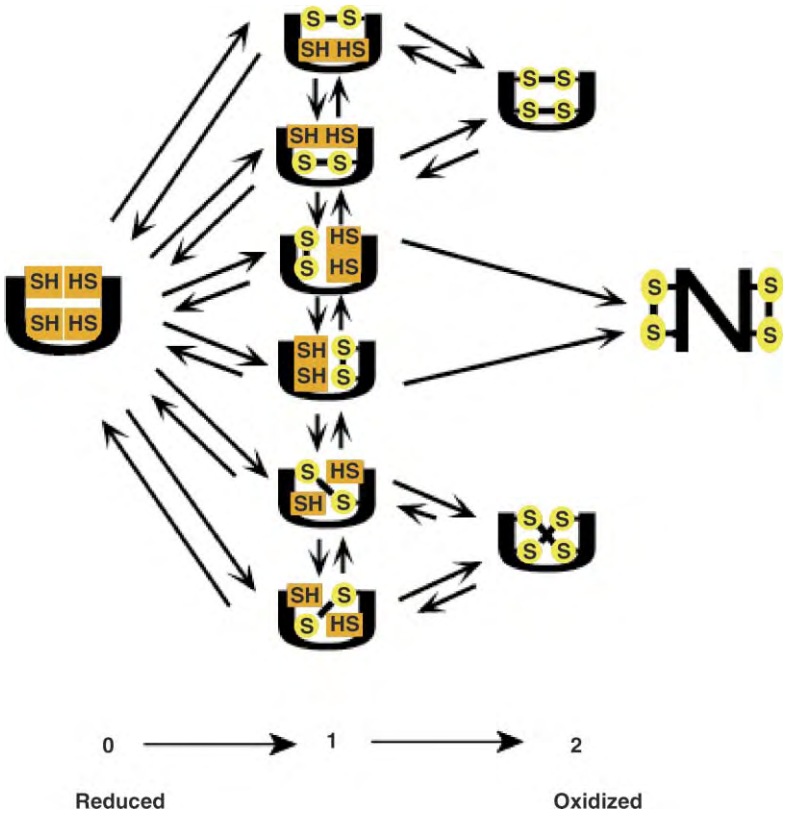



**FIGURE 3** Generalized mechanism of oxidative protein folding. The initial stages of oxidative folding are error-prone and often lead to the connection of cysteines that are not connected in the native structure. Different proteins will generate a different population of misoxidized species which are converted to the native structure (N) by disulfide bond isomerization.

slow (hours to days) to be biologically relevant. The resulting search for a biological catalyst for oxidative folding led to the discovery of the first catalyst of protein folding, protein disulfide isomerase (PDI).

## Protein Disulfide Isomerase

PDI accelerates the regain of native structure by catalyzing the formation of disulfide bonds and by providing a way to correct mistakes in connecting cysteines. PDI is an enzyme that has both oxidase (disulfide formation) and isomerase (disulfide rearrangement) activities. PDI accelerates thiol/disulfide exchange reactions (Figure 1) with proteins and small-molecule substrates. The final outcome of the reaction will depend on the redox conditions. The catalytic sites are contained in two thioredoxin homology domains located at each end of the PDI molecule. These active sites, both with the sequence CGHC, have the correct redox potential to effectively catalyze oxidation, reduction, and exchange reactions under the redox conditions of the endoplasmic reticulum. PDI interacts with its protein substrates but in a way that does not direct which cysteines are connected in the substrate. The mechanism involves several rounds of making and breaking disulfide bonds until the final structure becomes resistant to further isomerization of its disulfides.

## The Thioredoxin Family

Many of the thiol–disulfide exchange reactions of the cell are mediated by members of the thioredoxin family. This large family of proteins is characterized by an active site sequence of CXXC and an $\alpha$–$\beta$ secondary structure. The structure greatly affects the redox potential for reducing the active site disulfide. Thioredoxin, the patriarch of the family, is an excellent reducing agent involved in the biosynthesis of deoxyribonucleotides for DNA synthesis and in helping maintain reduced proteins in the cellular cytoplasm. DsbA, a periplasmic oxidase in bacteria is an excellent oxidizing agent that inserts disulfides into secreted proteins. The ease of forming the CXXC disulfide varies by a factor of over $10^5$, corresponding to a redox potential difference of more than 0.15 V.

## Disulfide Formation in the Cell

Disulfide bond formation state is influenced by the thiol/disulfide redox environment of the cellular compartment where the protein is located. Most disulfides are found in secreted or extracellular proteins where the environment is oxidizing. Generally, the cytoplasm of most cells, bacterial and eukaryotic, is very reducing, due to the high concentration of glutathione (GSH, a tripeptide, γ-glutamylcysteinylglycine) and the low concentration of glutathione disulfide.

### Extracellular Proteins

Proteins destined for the extracellular environment acquire disulfides after they are secreted from the cytoplasm during translation. In bacteria, an elaborate system of periplasmic and membrane-bound proteins, comprising the Dsb system, provides the periplasm with oxidizing equivalents to enable disulfide formation and reducing equivalents and catalytic isomerases to correct errors during protein folding (Figure 4). DsbB couples the bacterial electron transport system to disulfide formation, providing a source of oxidizing agent which are delivered directly to folding proteins by DsbA. Reducing equivalents to enable disulfide isomerization are provided by thioredoxin in the cytosol and transferred across the inner bacterial membrane by DsbD to DsbC, the periplasmic isomerase.

A similar system operates in eukaryotic cells, although all the elements of the system have not been found. Extracellular proteins are synthesized on the rough endoplasmic reticulum and translocated into the lumen (inside) during translation. The lumen of the endoplasmic reticulum is a more oxidizing intracellular compartment than the cytoplasm. Oxidizing equivalents are transferred from Ero1 to PDI and then to the folding proteins. The ultimate source of the oxidizing agent is not yet known. Reducing agents of the endoplasmic reticulum enable PDI and other catalysts to correct any errors in pairing cysteines by reducing the incorrect disulfide bond. Reducing agents are provided by glutathione, presumably by importing GSH from the cytoplasm. The endoplasmic reticulum has an elaborate quality control mechanism, which prevents misfolded proteins from exiting the endoplasmic reticulum or directs them to degradation pathways. The error-correction activity of PDI plays a critical role in this system. Depending on the details of the folding mechanism dictated by the primary sequence, different extracellular proteins may use different components of the complex folding and quality control apparatus of the endoplasmic reticulum to gain their correct three-dimensional structure, including the disulfide bonds that stabilize the structures.

### Disulfide Formation as a Regulatory Mechanism

Because disulfide bond formation is reversible, disulfide bonds can also regulate biological activity through their ability to stabilize specific protein structures. For example, the bacterial transcription factor, OxyR, senses the redox environment of the aerobic bacterial cell

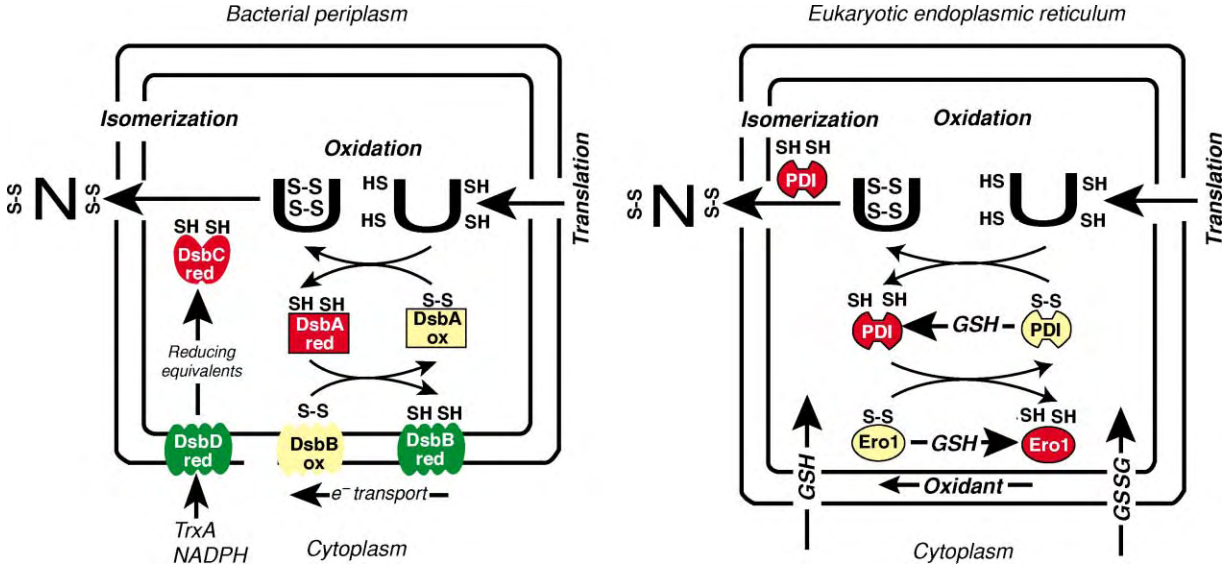



**FIGURE 4** Disulfide bond formation in bacterial and eukaryotic cells. In both cells, the major pathways for disulfide formation are located in specialized cellular compartments where the redox state of the environment is maintained in a more oxidized state than the cytosol.

through reversible disulfide bond formation linked to the cellular redox state and oxygen metabolism. Reduced OxyR is inactive as a transcription factor and upon oxidation and disulfide formation, the transcription factor can regulate the transcription of genes involved in the protection of the cell against oxidative stress and reactive oxygen species. Similar mechanisms operate in eukaryotic cells.

## See Also the Following Articles

Glycoprotein Folding and Processing Reactions • Protein Folding and Assembly

## Glossary

**bacterial periplasm** A specialized compartment in between the inner and outer membrane of bacteria. Disulfide formation occurs in this compartment.

**disulfide bond** A covalent bond between two sulfhydryl groups. In proteins, this bond covalently connects the sulfur atoms of two cysteine residues.

**endoplasmic reticulum** A specialized compartment of eukaryotic cells in which extracellular proteins are processed for secretion or inclusion in other cellular compartments.

**glutathione (GSH)** A tripeptide, $\gamma$-glutamylcysteinylglycine found at high concentrations in cells. It is the major thiol compound in most cells involved in redox reactions and the protection of cells against reactive oxygen species and toxic compounds.

**oxidation** Disulfide bond formation (formally, the loss of electrons) oxidation.

**oxidative folding** The coupling of disulfide formation to the folding of a protein into its correct three-dimensional structure. Disulfides stabilize protein structure and *vice versa*.

**protein folding** The process by which an unfolded protein gains its three-dimensional structure.

**reduction** Formally, the gain of electrons. Disulfide bonds are reduced to two thiols.

**thiol** A sulfhydryl (–SH) group.

## Further Reading

Collet, J.-F., and Bardwell, J. C. A. (2002). Oxidative protein folding in bacteria. *Mol. Microbiol.* **44**, 1–8.

Creighton, T. E. (1984). Disulfide bond formation in proteins. *Method Enzymol.* **107**, 305–329.

Frand, A. R., Cuzzo, J. W., and Kaiser, C. A. (2000). Pathways for protein disulphide bond formation. *Trends Cell Biol.* **10**, 203–210.

Gilbert, H. F. (1998). Protein disulfide isomerase. *Method Enzymol.* **290**, 26–50.

Gitler, C., and Danon, A. (eds.) (2003). *Cellular Implications of Redox Signaling*. Imperial College Press, London.

Goldberger, R. F., Epstein, C. J., and Anfinsen, C. B. (1963). Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. *J. Biol. Chem.* **238**, 628–635.

Helenius, A. (2001). Quality control in the secretory assembly line. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356**, 147–150.

Ritz, D., and Beckwith, J. (2001). Roles of thiol-redox pathways in bacteria. *Annu. Rev. Microbiol.* **55**, 21–48.

Scheraga, H. A., Konishi, Y., and Ooi, T. (1984). Multiple pathways for regenerating ribonuclease A. *Adv. Biophys.* **18**, 21–41.

Weissman, J. S., and Kim, P. S. (1991). Reexamination of the folding of BPTI: predominance of native intermediates. *Science* **253**, 1386–1390.

## Biography

Hiram F. Gilbert is a Professor of Biochemistry and Molecular Biology and Associate Dean of the Graduate School of Biomedical Sciences at Baylor College of Medicine in Houston, Texas. His principal research interests are in the catalysis of disulfide bond formation in the eukaryotic endoplasmic reticulum and the cellular function of disulfides. He holds a Ph.D. in Organic Chemistry from the University of Wisconsin, Madison and received his postdoctoral training at Brandeis University.

# DNA Base Excision Repair

**Hilde Nilsen and Tomas Lindahl**
*Cancer Research UK, London Research Institute, London, UK*

**DNA decomposes spontaneously as a result of chemical modifications inflicted by water and reactive by-products of normal aerobic metabolism. This spontaneous DNA base damage has both mutagenic and cytotoxic consequences. The quantitatively most important classes of such endogenous DNA damage are (1) loss of the purine bases guanine (G) and adenine (A) due to hydrolysis of the *N*-glycosyl bond between the DNA base and the backbone, leaving an abasic (AP) site in the DNA; (2) hydrolytic deamination of cytosine (C) and 5-methylcytosine (5-mC) to generate U:G and T:G base pairs, respectively; (3) formation of base oxidation products by reactive oxygen species, the most studied being 8-hydroxyguanine (8-oxoG) and ring-saturated pyrimidines, such as thymine glycol (Tg); (4) methylation of bases by nuclear methyl donors at reactive nitrogen or oxygen groups, one important product being the cytotoxic residue 3-methyladenine (3-mA); and (5) oxidation of nucleotide precursors resulting in the incorporation of damaged bases into DNA during semiconservative replication, such as the misincorporation of 8-oxodGMP to form 8-oxoG:A base pairs. DNA base excision repair (BER) is the main repair pathway for these lesions, both in the nucleus and in the mitochondria.**

## Core Base Excision Repair Factors

DNA base excision repair (BER) involves one damage-specific step to identify and release the damaged or, in some cases, mispaired base and a subsequent set of general steps that result in the reinsertion of one or a few nucleotides. There are three major modes of the DNA BER pathway that can be executed by a set of core factors (Figure 1). The choice of pathway is influenced by the initiating DNA glycosylase and the nature of the 3′ and 5′ residues generated during the repair process.

### DNA Base Excision

The damaged base is recognized by one of several DNA glycosylases (Table I), which initiate repair by hydrolyzing the *N*-glycosyl bond linking the base and the DNA backbone, leaving an AP site in the DNA. Notwithstanding limited sequence homologies, structural studies of the three-dimensional protein folds have revealed that DNA glycosylases can be divided into two main families. The mammalian members of the smaller family are UNG, TDG, and SMUG1. Members of the larger family are characterized as having a common structural motif and include the OGG1, MYH, NTH, and MBD4 enzymes. In addition, DNA glycosylases are classified as being either monofunctional or bifunctional, with the latter having an associated DNA lyase activity for chain cleavage at AP sites. Several DNA glycosylases can excise the same substrates, but the substrate specificities differ due to architectural diversity of the substrate-binding pockets of the enzymes. Furthermore, variations in biochemical properties, subcellular localization, and regulation suggest that their functions are not entirely overlapping.

#### Uracil-DNA Glycosylases

Human cells have at least four enzymes that can remove uracil from DNA (UNG, SMUG1, TDG, and MBD4). The UNG and SMUG1 enzymes are unusual in that they can remove uracil from single-stranded DNA as well as from double-stranded DNA. UNG is highly specific for uracil, with a very high turnover number, whereas SMUG1 has broader substrate specificity with lower turnover number. UNG accumulates at sites of active DNA replication in S-phase nuclei via interaction with PCNA and is the main enzyme responsible for post-replicative removal of uracil resulting from dUMP misincorporation. UNG may also be important for the removal of deaminated cytosine residues in single-stranded regions of DNA, in front of the replication fork, or in highly transcribed genes, but the relative contributions of UNG and SMUG1 in the repair of deaminated cytosine residues in general is not clear. In contrast, both TDG and MBD4 require double-stranded DNA as substrates and remove U or T resulting from the deamination of cytosine or 5-methylcytosine (5-mC). MBD4 contains a 5-mCpG binding domain in addition to the DNA glycosylase domain and is probably restricted to repair in a TpG or UpG context, whereas TDG has broader substrate specificity. These are enzymes with very low turnover numbers as a result of strong binding to AP sites.

 © 2004, Elsevier Inc. All Rights Reserved. 

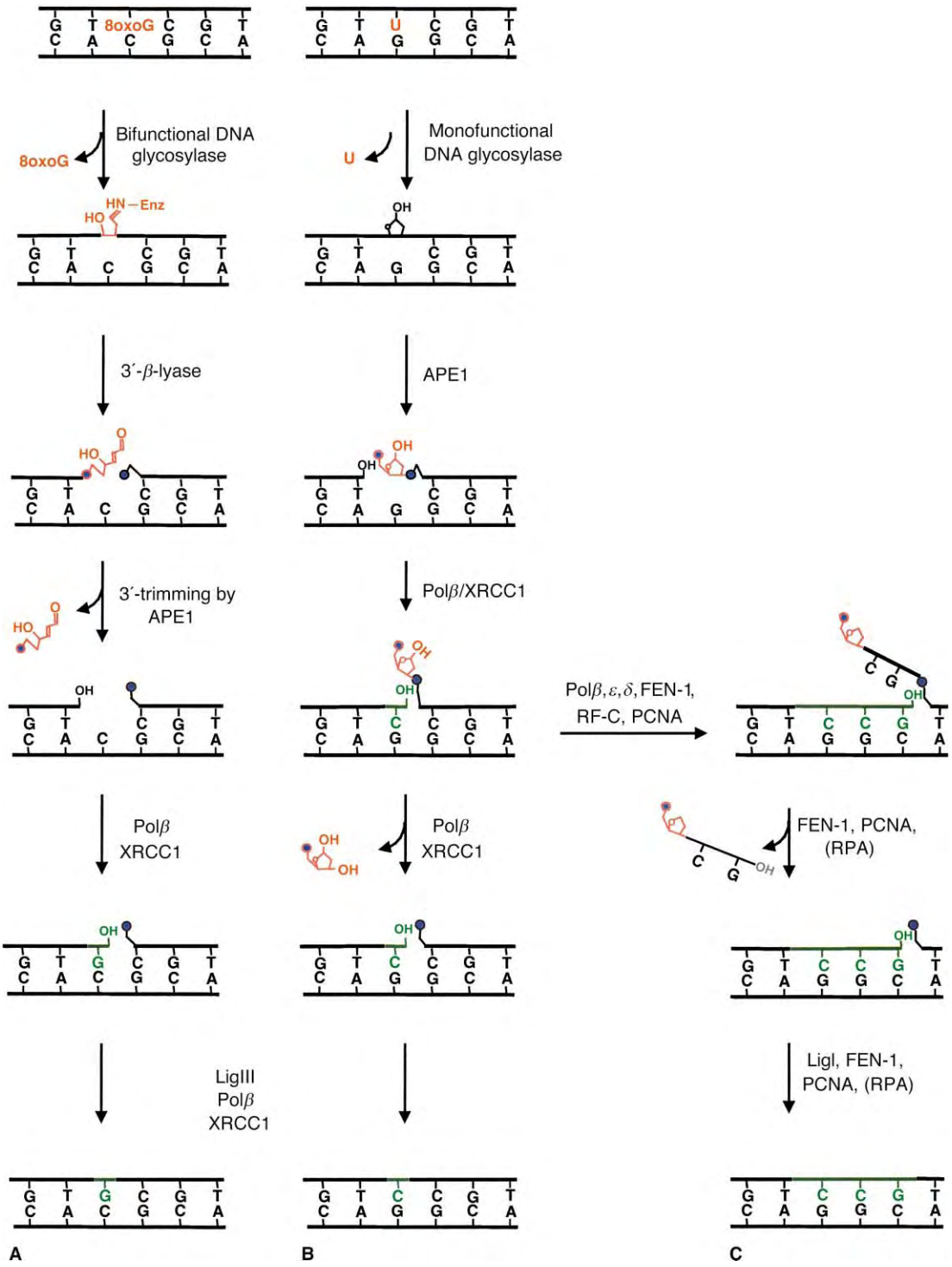



**FIGURE 1** DNA base excision repair pathways. (A) One nucleotide gap repair initiated by a bifunctional DNA glycosylase. (B) Short-patch repair is the major mode of repair initiated by a monofunctional DNA glycosylase. (C) Long-patch repair is used when short-patch repair is blocked. Reactive groups in each step are shown in red, phosphate groups are indicated as blue circles, and green blocks represent newly synthesized DNA. Adapted from Krokan, H. E., Nilsen, H., Skorpen, F., Otterlei, M., and Slupphaug, G. (2000) Base excision repair of DNA in mammalian cells. *FEBS Lett.* **476**, 73–77 and Klungland, A., Hoss, M., Gunz, D., Constantinou, A., Clarkson, S. G., Doetsch, P. W., Bolton, P. H., Wood, R. D., and Lindahl, T. (1999) Base excision repair of oxidative DNA damage activated by XPG protein. *Mol. Cell* **3**, 33–42.

**TABLE I**

**Human DNA Glycosylases and their Major Substrates**

| Enzyme name | Lyase activity | Cellular localization[a] | Major substrates[b] |
|---|---|---|---|
| UNG | No | N/M | U in single- or double-stranded DNA |
| SMUG1 | No | N | U in single- or double-stranded DNA |
| TDG | No | N | U, T or ethenoC opposite G |
| MBD4 | No | N | U or T opposite G in U/TpG context |
| MPG | No | N | 3-mA |
| MYH | No | N/M | A opposite 8-oxoG |
| OGG1 | Yes | N/M | 8-oxoG opposite C |
| NTH1 | Yes | N/M | Ring-saturated or fragmented pyrimidines |
| NEIL1 | Yes | N | Tg |
| NEIL2 | Yes | N | Oxidation products of C or U |
| NEIL3 | Yes | N | Fragmented or oxidized pyrimidines |

[a] The enzyme or specific isoforms of it are sorted to nuclei (N) or mitochondria (M).
[b] Only major substrates are shown. A comprehensive list of substrates for each DNA glycosylase can be found in Wood *et al.* (2001) and a regularly updated version can be found at that article's supplementary Web site.

### Methylpurine DNA Glycosylase

Methylpurine DNA glycosylase (MPG, also called 3-alkyladenine DNA glycosylase, AAG) is a monofunctional DNA glycosylase that structurally forms a class of its own. The major substrate for MPG is 3-methyladenine (3-mA) in double-stranded DNA, but the enzyme has an unusually wide substrate binding pocket that can accommodate a wide range of modified purine bases. Interestingly, MPG has a weak, but significant activity toward normal purine bases, illustrating the importance of tight regulation of individual BER enzymes.

### DNA Glycosylases for Removal of Oxidized Bases

The repair of oxidized DNA bases is predominantly initiated by bifunctional DNA glycosylases that use an amino group of the enzyme rather than a water molecule as a nucleophile to cleave the *N*-glycosyl bond. Enzyme-catalyzed resolution of the resulting Schiff-base intermediate, referred to as the AP-lyase activity, leads to the incision of the DNA backbone 3′ to the AP site. Several DNA glycosylases specific for oxidized DNA bases have been identified (Table I). Similar to the uracil-DNA glycosylases, they have different but overlapping substrate specificity. Oxidized purines are primarily removed by OGG1, whereas oxidized and fragmented pyrimidines are removed by the NTH1 and NEIL enzymes.

Despite having structural homology to the bifunctional DNA glycosylases, MYH acts as a monofunctional DNA glycosylase when removing unmodified adenine base-paired with 8-hydroxyguanine (8-oxoG). MYH colocalizes, via interaction with PCNA, with replication foci in the S-phase nuclei, where it removes adenine misincorporated opposite 8-oxoG during semi-conservative replication.

### Base-Flipping and Release

Despite the lack of sequence homology, DNA glycosylases show structural similarities, which allow base recognition and catalysis to proceed by related mechanisms. Pinching of the DNA backbone followed by intercalation of amino acid side chains into the DNA provides the basis for substrate recognition and catalysis. Consequently, the damaged nucleotide is flipped out of the helix and the scissile bond is exposed upon the stabilization of the base in the substrate-binding pocket. DNA glycosylases then release the damaged base after cleavage of the *N*-glycosyl bond.

## Backbone Incision

Following excision of the damaged base, BER can proceed through several routes. Subsequent to base excision by a monofunctional DNA glycosylase, the DNA backbone is incised 5′ of the AP site by AP-endonuclease 1 (APE1), creating a 3′OH group and a 5′-dRP moiety (Figure 1B). Upon resolution of the Schiff-base intermediate formed by a bifunctional DNA glycosylase, the DNA backbone is cleaved 3′ to the damage to give a 5′-phosphate and a blocked, 3′ $\alpha$, $\beta$ unsaturated aldehyde or a 3′-phosphate by elimination reactions. Although the NEIL enzyme domains deviate from the OGG1/NTH1 superfamily, they are believed to act by similar mechanisms. The blocked 3′ residue must

be removed to generate a 3′-OH group that is a substrate for DNA polymerases. This is achieved by the 3′ phosphodiesterase activity of APE1, leaving a one nucleotide gap in DNA (Figure 1A).

### Base Insertion

DNA polymerase $\beta$ (Pol$\beta$) is the main DNA polymerase involved in BER and comprises a polymerase domain and a dRP-lyase domain. The 3′-OH group is a substrate for the polymerase function of Pol$\beta$. Pol$\beta$ is distributive and will only synthesize a few nucleotides before disengaging. If the 5′-dRP moiety is a substrate for the dRP-lyase activity of Pol$\beta$, then one or two nucleotides will be inserted in concert with the removal of the dRP moiety and BER will proceed through the short-patch pathway (Figure 1B). If, however, the dRP moiety is modified, making it a poor substrate for the dRP-lyase activity of Pol$\beta$, this polymerase may still incorporate one nucleotide, but BER will then be funneled into the long-patch pathway, which uses DNA replication factors to synthesize a longer repair patch (Figure 1C). In this case, DNA polymerase $\delta/\varepsilon$ performs strand-displacement synthesis, supported by replication factor C (RF-C) and proliferating cell nuclear antigen (PCNA), and the modified dRP moiety is removed as part of an oligonucleotide released upon Flap endonuclease 1 (FEN1) incision. Finally, because a 1-nucleotide gap in DNA is a poor substrate for the strand-displacement activity of Pol$\beta$, a single nucleotide will be inserted when repair is initiated by a bifunctional DNA glycosylase (Figure 1A). Oxidative base lesions frequently occur in clusters, and this pathway might have evolved to prevent the collision between closely spaced repair tracts on different strands, which otherwise could generate double-strand breaks.

Pol$\beta$ lacks the editing 3′-exonuclease function characteristic of replicative DNA polymerases. Consequently, the accuracy of DNA synthesis by Pol$\beta$ is relatively low. In the case of BER, the proofreading required is probably performed by a cryptic 3′-exonuclease activity of the APE1 enzyme.

### Nick Rejoining

Both DNA ligase III (LigIII) and DNA ligase I participate in BER. Pol$\beta$ interacts directly with X-ray cross complementing factor 1 (XRCC1). XRCC1 also interacts with LigIII, and this interaction stabilizes LigIII. Pol$\beta$, XRCC1, and LigIII form a trimeric complex on substrate DNA, and LigIII is the main nick rejoining activity for short-patch and 1-nucleotide gap repair. DNA ligase I predominantly seals nicks after long-patch repair via direct protein–protein interaction with PCNA.

### Pathway Orchestration

One characteristic of the BER pathway is the highly orchestrated manner with which proteins interact sequentially on the DNA substrate, as opposed to the assembly of large preformed nuclear complexes. The DNA glycosylases all remain bound at AP sites, albeit to varying degrees, but are efficiently displaced by APE1. Pol$\beta$ is in turn recruited through interaction with APE1, and the trimeric complex of DNA Pol$\beta$-XRCC1-LigIII ensures that the repair intermediates are protected. There is a progressive bending of the DNA backbone during the repair pathway, starting from a 30° kink when the DNA glycosylase is bound to the substrate, progressing to 45° (APE1-bound), and then becoming almost a 90° bend when the trimeric Pol$\beta$-XRCC1-LigIII is bound. Progressive bending and pairwise protein–protein interactions probably aid substrate recognition and give directionality to the pathway without exposure of cytotoxic repair intermediates, such as AP sites and dRP moieties, thus preventing aberrant processing of intermediates and premature activation of cell cycle checkpoints in response to DNA damage.

## Base Excision Repair in Chromatin

The organization of DNA into chromatin in eukaryotic cells restricts access for several DNA binding proteins to their substrates and introduces steric constraints that might hamper catalytic reactions on the nucleosome surface. Although BER proceeds more slowly in chromatin than in free DNA, the core BER proteins are apparently able to access DNA on the surface of a nucleosome. Chromatin remodeling might therefore not be a prerequisite for repair of DNA in nucleosomes, but it would presumably facilitate repair in chromatin. Furthermore, mammalian cells might circumvent a need for rapid DNA repair in condensed chromatin by coupling BER to other DNA transactions where the chromatin structure is relaxed. This would ensure efficient repair when speed is of importance, for example, in the cases of UNG and MYH, by targeting the enzymes to sites of active replication.

## Base Excision Repair in Mitochondria

Being close to the centers for oxidative metabolism in the cell, mitochondrial DNA is particularly exposed to reactive oxygen species. Several alternatively spliced forms of DNA glycosylases have been identified, and some isoforms are specifically sorted to mitochondria (denoted M in Table I). APE1 is transported to mitochondria and the mitochondrial DNA polymerase

$\gamma$ has dRP-lyase activity. mtDNA ligase is a processed form of DNA LigIII. Thus, the mitochondria have all the activities required for BER, and BER might be the most important mode of DNA repair in this organelle.

## The Cancer Connection

Bacterial and yeast mutants deficient in DNA glycosylases often exhibit elevated spontaneous mutation frequencies. Despite an observed accumulation of DNA base damage in gene-targeted knockout mice, they generally show weaker phenotypes in this regard, presumably because of the high degree of functional overlap among different DNA glycosylases (Table I) and the existence of several efficient and specialized DNA polymerases for translesion synthesis. There are at least four mammalian DNA glycosylases that can remove uracil from DNA and, although Ung-deficient mice accumulate uracil in the genome, they show only a modest increase (less than 50%) in the mutation frequency of a transcriptionally inactive transgene. Nevertheless, the Ung-deficient mice are susceptible to the spontaneous development of B-cell lymphomas. No spontaneous tumor development has been observed in other DNA glycosylase deficient mice, but Mbd4-deficiency accelerates tumor development in a tumor-prone mouse strain. However, human polymorphisms have been identified in most BER genes, and mutations in MYH have been linked convincingly to a higher susceptibility to colorectal cancer.

## Base Excision Repair Branching Out

Interestingly, attempts to generate gene-targeted knockout mice for genes downstream of the DNA glycosylases result in embryonic lethality. Similarly, genetic studies with budding yeast have shown that mutants unable to repair AP sites in DNA are not viable. This could be a result of the accumulation of cytotoxic repair intermediates, AP sites or dRP moieties in particular, or a consequence of the involvement of these proteins in other cellular pathways. BER factors associated with the long-patch pathway exert their main function during DNA replication, but other BER factors may also be employed in DNA transactions in addition to classical BER, for example, the involvement of Pol$\beta$ in meiosis. Mammalian APE1 has an N-terminal domain that allows it to function as a redox factor; this domain is required for redox activation of various spontaneously oxidized transcription factors, such as AP1. Recently, the Ung uracil-DNA glycosylase was shown to be central in the diversification of immunoglobulin genes, a finding that opens up new and exciting prospects for research into the biological significance of DNA BER.

## See Also the Following Articles

Chromatin: Physical Organization • DNA Glycosylases: Mechanisms • DNA Polymerase $\beta$, Eukaryotic • DNA Polymerases: Kinetics and Mechanisms • Mitochondrial DNA • Nuclear Organization, Chromatin Structure, and Gene Silencing • Nucleotide Excision Repair and Human Disease • Nucleotide Excision Repair, Bacterial: The UvrABCD System • Nucleotide Excision Repair: Biology • Nucleotide Excision Repair in Eukaryotes

## Glossary

**cytotoxic DNA base** A DNA base that blocks the progression of replication forks or transcription.

**hydrolysis** A chemical reaction involving a water elimination reaction; a reaction in which a molecule decomposes to two molecules, one smaller than the other.

**mutagenic DNA base** A DNA base that introduces changes in the coding sequence of DNA.

**Schiff base** A covalent compound formed by a condensation reaction between an aromatic amine in the enzyme and an aldehyde or ketone of the deoxyribose group of DNA.

## Further Reading

Bogenhagen, D. F., Pinz, K. G., and Perez-Jannotti, R. M. (2001). Enzymology of mitochondrial base excision repair. *Prog. Nucleic Acid Res. Mol. Biol.* **68**, 257–271.

Friedberg, E. C., and Meira, L. B. (2000). Database of mouse strains carrying targeted mutations in genes affecting cellular responses to DNA damage: Version 4. *Mutat. Res.* **459**, 243–274.

Friedberg, E. C., Walker, G. C., and Siede, W. (1995). *DNA Repair and Mutagenesis.* ASM Press, Washington D.C.

Hosfield, D. J., Daniels, D. S., Mol, C. D., Putnam, C. D., Parikh, S. S., and Tainer, J. A. (2001). DNA damage recognition and repair pathway coordination revealed by the structural biochemistry of DNA repair enzymes. *Prog. Nucleic Acid Res. Mol. Biol.* **68**, 315–347.

Klungland, A., Hoss, M., Gunz, D., Constantinou, A., Clarkson, S. G., Doetsch, P. W., Bolton, P. H., Wood, R. D., and Lindahl, T. (1999) Base excision repair of oxidative DNA damage activated by XPG protein. *Mol. Cell* **3**, 33–42.

Krokan, H. E., Nilsen, H., Skorpen, F., Otterlei, M., and Slupphaug, G. (2000) Base excision repair of DNA in mammalian cells. *FEBS Lett.* **476**, 73–77.

Lindahl, T. (2001). Past, present and future aspects of base excision repair. *Prog. Nucleic Acid Res. Mol. Biol.* **68**, 17–30.

Rada, C., Williams, G. T., Nilsen, H., Barnes, D. E., Lindahl, T., and Neuberger, M. S. (2002). Immunoglobulin isotype switching is inhibited and somatic hypermutation perturbed in Ung-deficient mice. *Curr. Biol.* **12**, 1748–1755.

Wood, R. D., Mitchell, M., Sgourous, J., and Lindahl, T. (2001). Human DNA repair genes. *Science* **291**, 1284–1289.

Wood, R. D., Mitchell, M., Sgourous, J., and Lindahl, T. Human DNA repair genes supplement. Available at: http://www.cgal.icnet.uk/DNA_Repair_Genes.html.

## Biography

Dr. Hilde Nilsen is a postdoctoral research fellow at the Cancer Research U.K., London Research Institute. Her research interests are cellular responses to spontaneous DNA damage. She received her Ph.D. from the Norwegian University of Science and Technology in Norway. She has been awarded fellowships from EMBO and the EU Marie Curie program.

Dr. Tomas Lindahl is Director of Research at the Clare Hall Laboratories of the Cancer Research U.K., London Research Institute. His research interests are in DNA repair of spontaneous DNA damage and genome instability. He has an M.D. from the Karolinska Institute, Stockholm. He is a member of EMBO, a fellow of the Royal Society, and a member of the Swedish and Norwegian Academies of Science, and he has published extensively over a period of 30 years.

# DNA Damage: Alkylation

**Anton B. Guliaev and B. Singer**
*Lawrence Berkeley National Laboratory, Berkeley, California, USA*

**Alkylation is the reaction of electrophilic chemical compounds or alkylating agents with the nucleophilic centers in organic macromolecules. Alkylating agents include a large variety of chemicals, some of which are potent mutagens or carcinogens. It is well known that DNA is a key structure that determines development and reproduction of the leaving organisms ranging from simple cells to humans. The sequence and structure of individual nucleotides in DNA are essential for the high degree of fidelity required for generating a "blueprint" during the process of the storing and passing of genetic information from one organism to another. However, DNA is subject to alteration in structure and/or sequence of individual bases. These changes can result in errors during the steps of the DNA replication, recombination, and repair, thus leading to the modification of the molecular structure of genetic material. Most of these modifications can be successfully removed by the DNA repair machinery. However, a significant amount of DNA remains unrepaired, which can lead to mutagenesis.**

**The common source of the modifications in DNA, particularly the alteration in the nucleotide structure, can be found in the instability of specific chemical bonds of nucleotides under different physiological conditions, such as temperature or pH. Moreover, the DNA structure readily reacts with multiple chemical compounds and physical agents found in the environment. These mutagens and carcinogens can be produced by a variety of chemical reactions, metabolism of other living forms, and they also can be manmade. Modifications of the DNA molecular structure can be characterized as DNA damage. DNA damage is unavoidable and can be divided into two categories: (1) spontaneous, such as formation of mismatches, deamination of bases, loss of base, oxidative damage; (2) environmental, such as ionizing radiation, UV radiation, and chemical agents, including ubiquitous alkylating agents. DNA alkylation, which is a part of the DNA damage, is the reaction of the alkylating agents with the nucleophilic centers, such as oxygen or nitrogen in the DNA base or backbone. The result of the reaction is the formation of the modified DNA bases called *adducts* or formation of the phosphotriesters in the case of the interaction of the agent with the oxygen in the DNA phosphodiester backbone. The adduct together with the opposite base is usually called a *lesion*.**

## Alkylating Agents and Mechanism of the Reaction with DNA

The first chemical evidence of alkylation damage to DNA was reported in 1962 by the observation of 7-methylguanine as an *in vivo* product of dimethylnitrosamine administered to rats. Since that time, the list of alkylation agents and sites of alkylation has significantly increased, so that now we know that almost all nitrogens and oxygens of nucleotides in DNA can be modified. Alkylating agents include a large number of molecules, which can efficiently react with DNA causing structural modification of the base and phosphate group in DNA. The most common agents are alkyl sulfates, alkyl sulfonates, alkyl halides, dialkyl nitrosamine, alkyl nitrosoureas, acyl nitrosamides, mustards, diazo compounds, lactones, epoxides, etc. (Figure 1). These agents can be divided into two major groups: monofunctional and bifunctional. The monofunctional agents have only one reactive group that is involved in covalent interaction with the single center on DNA. The bifunctional agents have two reactive groups and have the ability to react with two centers on DNA. If the two centers are on the opposite strands of the DNA, the reaction of a bifunctional agent, such as sulfur or nitrogen mustards, can produce an interstrand cross-link. Many of the alkylating agents have a mutagenic effect and are known or suspected carcinogens. The reaction specificity of these compounds is different with different bases. Diazoalkanes react readily with guanosine and thymidine, but only under extreme conditions with adenosine and cytosine. In contrast, alkyl sulfates, alkyl sulfonates, mustards, epoxides, and nitroso compounds alkylate bases in the following order: $G > A > C \gg T$. The rate of ethylation is much slower than that of methylation for all classes of alkylating agents except alkyl iodides, which readily alkylate G, A and C.

The main chemical mechanism of DNA alkylation is a $S_N2$ and $S_N1$ type reaction in which the electrophile (alkylating agent) reacts with the electron-rich regions of the base residues and phosphodiester

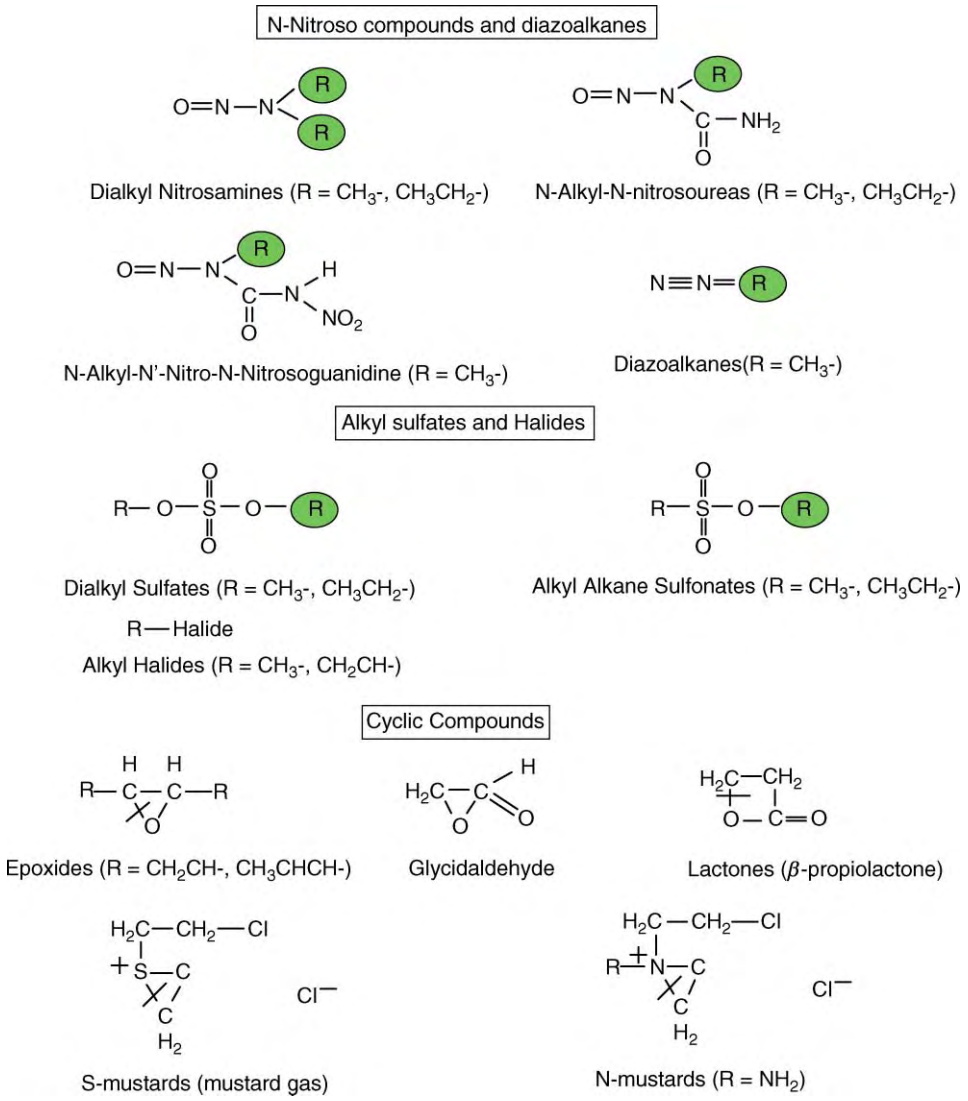
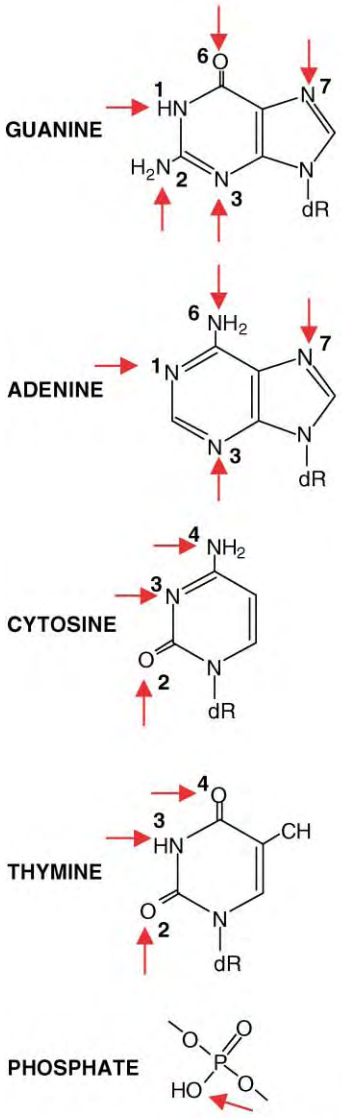



**FIGURE 1** Structural formulas of alkylating agents. The alkyl groups are indicated by R. Circled R shows which group of the molecule is being transferred. The opening of the ring for the cyclic compounds is indicated by the line, and the whole molecule acts as a substituent.

backbone. Figure 2 shows possible sites of alkylation on the DNA bases and the relative reactivity of these sites as measured by the presence of the alkylated base after the reaction with carcinogenic alkylating agents. The ring nitrogens, being more nucleophilic than the oxygens, are known to be more reactive with the alkylating agents. Thus, the N7 of guanine and N3 of adenine are the most reactive. The DNA phosphodiester backbone can also undergo an alkylation reaction through interaction of the agent with the oxygen, which results in the formation of phosphotriesters (Figure 2). However, not all alkylating agents react directly with the DNA bases. Some of them, such as dialkylnitrosamines or vinyl chloride (group of R-halides), must be metabolically activated.

## Effect of DNA Conformation on the Alkylation

There are at least two significant differences between alkylation of nucleosides and polynucleotides. One is the presence of the phosphate in the phosphodiester bonds, which can form phosphotriesters, and the other is the effect of secondary structure on the availability of reactive sites. In general, the major sites of alkylation of single-stranded synthetic polynucleotides are the same as those for nucleosides, but the extent of reaction is different. The neighboring base or the overall charge of the nucleotide can affect the nucleophilic potential of the DNA base. For example, if the guanine residue is flanked by another guanine, the negative electrostatic




| Adduct \ Carcinogenic alkylating agent | Percentage of total alkylation: DMN MNU SDMH | DEN ENU | MM S |
|---|---|---|---|
| $N^3$-Alkylguanine | 0.6 | 1.5 | 0.7 |
| $O^6$-Alkylguanine | 3–6 | 8 | 0.3 |
| $N^7$-Alkylguanine | 69 | 12 | 8.3 |
| $N^1$-Alkyladenine | 0.8 | 0.1 | 1.2 |
| $N^2$-Alkyladenine | 4 | 4 | 11 |
| $N^7$-Alkyladenine | 1.5 | 0.6 | 1.9 |
| $O^2$-Alkylcytosine | 0.1 | 2 | |
| $N^3$-Alkylcytosine | 0.5 | 0.3 | |
| $O^2$-Alkylthymine | 0.1 | 7 | |
| $N^3$-Alkylthymine | 0.3 | 0.4 | |
| $O^4$-Alkylthymine | 0.1 | 2.5 | |
| Triester | 12 | 58 | 1 |

DMN – Dimethylnitrosamine
MNU – Methylnitrosourea
SDMN – 1,2-Dimethylhydrazine
DEN – Diethylnitrosamine
ENU – Ethylnitrosourea
MMS – Methyl methanesulfonate

**FIGURE 2** Structural formulas of the DNA bases with the possible sites of the modification by alkylating agents indicated by red arrows. dR indicates sugar. The table on the right shows the percentage of total alkylation after reaction with the carcinogenic alkylating agent. The data presented in the table are available only for the subset of the sites shown.

potential of the N7 position of guanine is enhanced, thus providing a better environment for the electrophilic attack of the alkylating agent. However, the increase of the negative charge of the alkylating moiety diminishes the nucleophilic potential of the base. Steric effects also play an important role in the reaction between the alkylating agents and the nucleophilic sites in DNA. For the normal right-handed (B-form) helix conformation, the access to sites in DNA differs between the major and minor groove. For the guanine residue in the B-form DNA the O6 and N7 atoms are in the major groove, which can be easily accessed by the alkylating agent. In contrast, the relatively reactive N3 of the adenine lies in the minor groove, which is less accessible due to the steric implications of the B-form DNA.

## Methods of Identification of Alkyl Derivatives of Nucleic Acids

The initial identification and characterization of the alkylation products can be made using UV absorption

spectroscopy. The nature of the alkyl group introduced to the base does not affect the $\lambda_{max}$ or $\lambda_{min}$ significantly. However, the position of the base modification results in the spectral changes that make it possible to distinguish among derivatives. In addition to spectra maxima and minima of the cationic and anionic forms, the shape of the spectra changes significantly depending on the base modification. The characteristic shoulders and alteration in the curve profile can be assigned to a particular adduct. Additional identification can be performed using other methods such as mass spectrometry, nucleic magnetic resonance, and infrared spectroscopy. The detailed description of these methods and their use for identification of alkyl derivatives can be found in the Further Reading.

## Cytotoxic and Mutagenic Effects of Alkylation: Methylation

Mutation can be described as a change of one base to another, leading to a change in coding information. The change in the base structure, which is lethal to the cell, can be characterized as a cytotoxic.

Direct alkylation of DNA, particularly methylation, acting through the covalent modification of the base, has the ability to generate miscoding base derivatives and lesions that block replication. The major adducts generated in double-stranded DNA by methylating agents such as methyl methanesulfonate (MMS), dimethylsulfate (DMS) and methyl iodide (MeI)

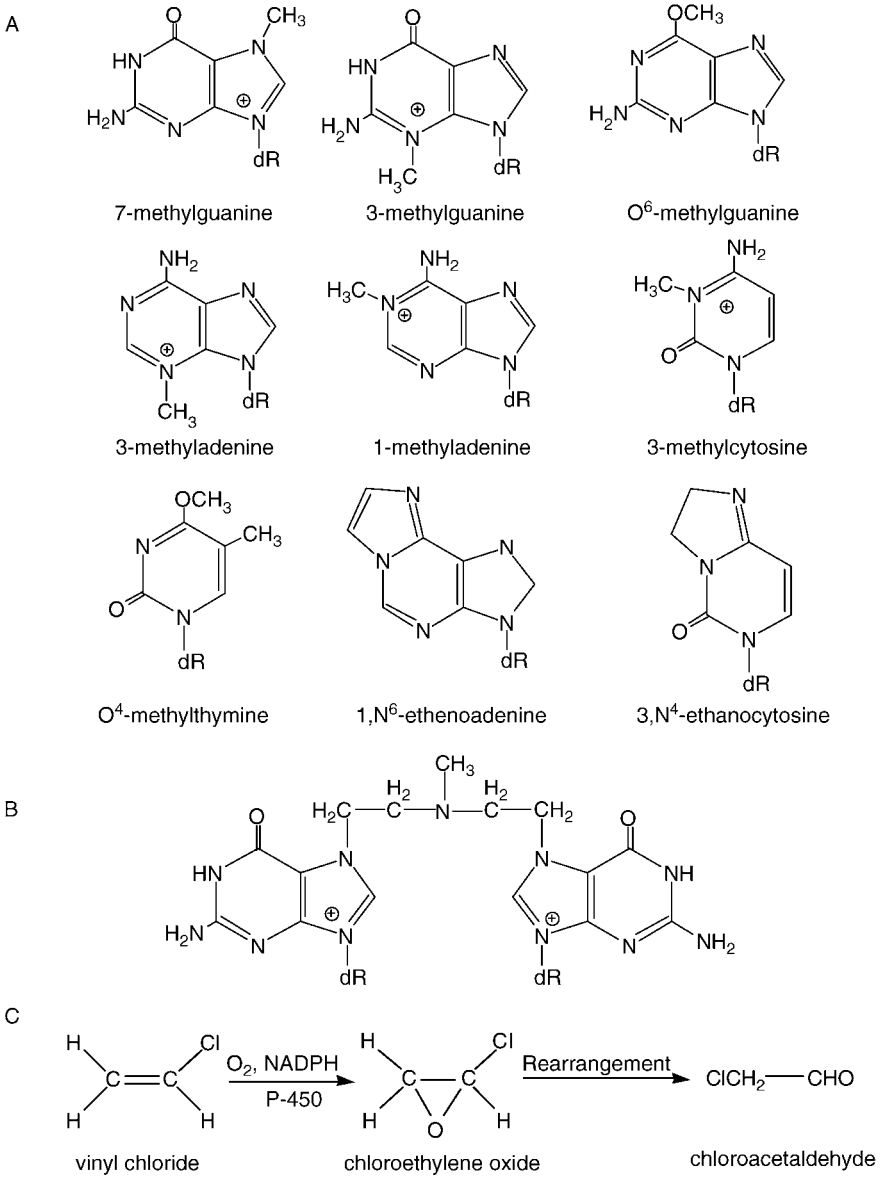



**FIGURE 3** (A) Selective structures of the DNA adducts formed by the alkylation reaction. (B) Intrastrand cross-link product of the reaction of nitrogen mustard with N-7 of guanine. (C) Mechanism of the metabolism of vinyl chloride.

are: 7-methylguanine (7-meG), 3-methyladenine (3-meA), 3-methylguanine (3-meG), and $O^6$-methylguanine ($O^6$-meG) (Figure 3A). In single stranded DNA, these methylating agents form 1-methyladenine (1-meA) and 3-methylcytosine (3-meC) (Figure 3A). The formation of these adducts in the single-stranded, but not in double-stranded DNA is due to the fact that these modification sites are involved in base-pairing and are therefore protected from alkylation. Adducts 3-meA, 3-meG and 3-meC have the ability to block DNA replication, thus having cytotoxic effects. In contrast, $O^6$-meG and $O^4$-methylthymine ($O^4$-meC) are miscoding base derivatives that mispair during replication thus leading to possible mutations. Environmental mutagens such as 1,2-dimethylhydrazine, diazoquinones, and tert-butylhydroperoxide generate methyl radicals that readily react with the guanine residue to form 8-methylguanine, which also has a high miscoding potential. However, the most common methylation product in DNA, 7-meG, is not mutagenic and basepairs normally in polynucleotides. Most of the adducts produced by methylation can be efficiently removed by a variety of DNA glycosylases through the base excision repair mechanism (BER). This process involves cleavage of a base–sugar bond in order to release the modified base and generate an apurinic/apyrimidinic site that is repaired by endonucleases. This is followed by the action of the DNA polymerase that incorporates the correct base. In contrast, O6-methylguanine is corrected by the direct transfer of the methyl group to a cysteine residue of the methyltransferase repair enzyme. Intrastrand cross-links (Figure 3B), generated by bifunctional alkylating agents, such as nitrogen mustard, represent an important class of DNA damage, since they prevent DNA strand separation, which is crucial to the processes of replication and transcription. The cell has the ability to repair this damage through the mechanism of nucleotide excision repair (NER), which uses multiple enzymes.

The nitroso compounds and hydrocarbons, which are well-known environmental carcinogens, react through a number of metabolic intermediates. However, the most effective direct alkylating agents, such as alkyl sulfates (Figure 1), in turn, are poor carcinogens. In contrast, the metabolically activated alkylating agents are very efficient carcinogens. One of the widely used common chemicals, which is carcinogenic in man and experimental animals, is vinyl chloride (Figure 3C). The industrial use of vinyl chloride is estimated to be about $4 \times 10^9$ kg/year in the United States. The exposure to vinyl chloride is generally by inhalation and results in angiosarcoma of the liver or other tumors in the brain, lung, and hematolymphopoietic system in humans. Vinyl chloride is readily metabolized into chloroethylene (CEO) oxide, which rapidly changes to chloroacetaldehyde (CAA) (Figure 3C). Both CEO and CAA are highly mutagenic and carcinogenic and react with the DNA bases to form etheno derivatives (e.g., 1,$N^6$-ethenoadenine and 3,$N^4$-ethanocytosine (Figure 3A)). These adducts have a high miscoding potential and also an ability to block DNA replication.

## See Also the Following Articles

DNA Base Excision Repair • Nucleotide Excision Repair, Bacterial: The UvrABCD System • Nucleotide Excision Repair in Eukaryotes

## Glossary

**adduct** Structurally modified DNA nucleotide.

**alkylating agent** Chemical compound that has the ability to react with the DNA through the reaction of alkylation.

**DNA** Deoxyribonucleic acid.

**nucleoside** Compound that consists of a purine or pyrimidine base linked to a pentose.

**nucleotide** Compound that consists of a nitrogeneous base, a sugar, and one or more phosphate groups.

## Further Reading

Lindahl, T., and Sedgwick, B. (1988). Regulation and expression of the adaptive response to alkylating agents. *Ann. Rev. Biochem.* **57**, 133–157.

Sedgwick, B., and Lindahl, T. (2002). Recent progress on the ada response for inducible repair of DNA alkylation damage. *Oncogene* **21**, 8886–8894.

Singer, B. (1975). The chemical effects of nucleic acid alkylation and their relation to mutagenesis and carcinogenesis. *Prog. Nucleic Acid Res. Mol. Biol.* **15**, 219–284.

Singer, B., and Grunberger, D. (1983). *Molecular Biology of Mutagens and Carcinogens*. Plenum Press, New York.

## Biography

B. Singer is a Research Professor Emeritus at the Virus Laboratory and Department of Molecular Biology at the University of California, Berkeley; she is currently Senior Scientist at Lawrence Berkeley National Laboratory. She has coauthored the text, "Molecular Biology of Mutagens and Carcinogens," as well as numerous papers on chemistry, molecular biology, and virology. In 1957, together with her husband, H. Fraenkel-Conrat, they discovered that all the genetic information for tobacco mosaic virus was coded by RNA alone. Her current research interests are in the chemical reactions of environmental carcinogens.

Anton B. Guliaev is a Physicist Scientist at Lawrence Berkeley National Laboratory, Berkeley, California.

# DNA Glycosylases: Mechanisms

**Daniel J. Krosky and James T. Stivers**
*The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA*

**The highly accurate replication of an organism's DNA is necessary for it to maintain genetic stability over many generations. Thus, cells need to aggressively repair DNA damage in order to prevent mutations and to eliminate toxic base modifications that can interfere with DNA replication. To this end, cells have evolved several DNA repair pathways geared toward processing different types of DNA lesions. One of these systems, the base excision repair (BER) pathway, recognizes and removes chemically modified DNA bases and replaces them with the correct nucleotides. The first step in BER is catalyzed by a family of functionally related enzymes known as DNA glycosylases that hydrolytically cleave the glycosidic bond between the damaged base and its deoxyribose sugar.**

## The Biological Function of DNA Glycosylases

DNA bases can be damaged by a variety of mechanisms that include alkylation, deamination, oxidation, and ultraviolet light, each of which produces a different base alteration. Repair of each of these diverse lesions begins with the action of a unique DNA glycosylase (to date, eight have been identified in humans) that is specific for the particular damaged base. This hydrolysis reaction results in a common intermediate, an abasic site, which can be processed by either short- or long-patch repair. In short-patch repair, only the damaged nucleotide is replaced, whereas in long-patch repair, the damaged base and three or four additional nucleotides are excised and replaced.

## Types of DNA Glycosylases

### Substrate Range and Specificity

The DNA glycosylases have evolved varying degrees of substrate specificity. For example, uracil–DNA glycosylase (UDG) will efficiently cleave uracil that has been misincorporated into DNA, but not thymine, which only differs from uracil by one methyl group. Other DNA glycosylases not only possess high specificity for the cleaved base, but also for the context in which it appears. Examples of these include the bacterial mismatch-specific UDG (MUG), which excises uracil paired with guanine but not with adenine, and the mammalian 8-oxoguanine glycosylase (OGG), which only cleaves the oxidized base 8-oxoguanine when it is opposite cytosine.

In contrast, other DNA glycosylases cleave a relatively wide spectrum of chemically related damaged bases. For instance, *Escherichia coli* 3-methyladenine glycosylase II (AlkA) catalyzes the hydrolysis of a wide range of cationic *N*-alkylated purines, such as 3-methyladenine and 7-methylguanine. In addition, AlkA can cleave undamaged purine bases, although with much lower efficiency than *N*-alkylated purines. Other broad-specificity glycosylases include mammalian methylpurine glycosylase (MPG), which has a substrate range similar to AlkA, and endonuclease III (Endo III), which processes a variety of oxidized pyrimidines (e.g., thymine glycol and 5-hydroxycytosine).

In addition to simply excising damaged DNA bases resulting in an abasic site, a subset of bifunctional DNA glycosylases can subsequently catalyze an elimination reaction in which the 3′-phosphate of the abasic site is expelled. Members of this bifunctional DNA glycosylase family include OGG, Endo III, and pyrimidine dimer DNA glycosylase (PDG). Other enzymes in the base excision repair (BER) pathway then process the resulting repair intermediate, mostly via the short-patch pathway.

### Structural Superfamilies of DNA Glycosylases

Even though DNA glycosylases catalyze similar reactions, these enzymes share minimal primary sequence homology with one another. However, X-ray crystallography and nuclear magnetic resonance (NMR) analyses have revealed that many of these proteins share a common fold and have allowed the classification of these proteins into several structural superfamilies. One of these structural superfamilies is defined by the helix-hairpin-helix (HhH) motif, which is found in many non-sequence-specific DNA-binding proteins. This $\alpha$-helical fold allows these enzymes to make

 © 2004, Elsevier Inc. All Rights Reserved. 

nonspecific contacts with the DNA phosphodiester backbone, thus permitting sequence-independent excision of their cognate lesions. Other DNA glycosylases, such as UDG, have evolved alternate structural motifs that also result in nonspecific DNA binding and fufill the same function as the HhH domain.

## Functional Commonalities of DNA Glycosylases

### Base Flipping

Despite differences in substrate specificity, structure, and primary sequence, all DNA glycosylases are functionally related in their ability to extrude DNA bases from the double helix and to catalyze the hydrolysis of the glycosidic bond between the cognate base and the deoxyribose. Extruding a base from duplex DNA, or base flipping, is the process by which an enzyme facilitates the rotation of the DNA phosphodiester backbone such that a base flips from within the double helix to an extrahelical position located within the binding pocket of the enzyme (Figure 1). The most common mode of base flipping occurs when the enzyme flips the damaged base from the DNA double helix. However, some DNA glycosylases use different base-flipping strategies. PDG, which is responsible for cleaving the glycosidic bond of thymine photodimers, flips the adenine that is opposite the target base. *E. coli* MutY, which removes adenine misincorporated opposite to the oxidized base 8-oxoguanine, appears to flip both bases from the duplex.

There are several elements common to the base-flipping mechanisms of DNA glycosylases. First, the enzyme makes extensive nonspecific contacts with the phosphodiester backbone of the DNA (Figure 1). In addition to allowing sequence-independent binding of the DNA glycosylase to DNA, these protein–DNA interactions provide a stable architecture from which the enzyme can distort the DNA. All structural studies of DNA glycosylases bound to DNA have revealed that the DNA is significantly bent at the site of enzyme binding, albeit to differing degrees with different enzymes (Figure 1). Although the exact role of DNA bending is unclear, it may help certain glycosylases locate lesions in DNA. In addition, computational studies have suggested that DNA bending may play an important role in lowering the activation barrier for base flipping, thus providing an explanation for why DNA bending is such a ubiquitous feature of these interactions (Figure 1). Finally, all DNA glycosylases insert a bulky amino acid side chain, such as leucine or phenylalanine, inside the DNA double helix (Figure 1). This group serves as a structural wedge to both push the base out and to act as a barrier to prevent the base from slipping back into the double helix.

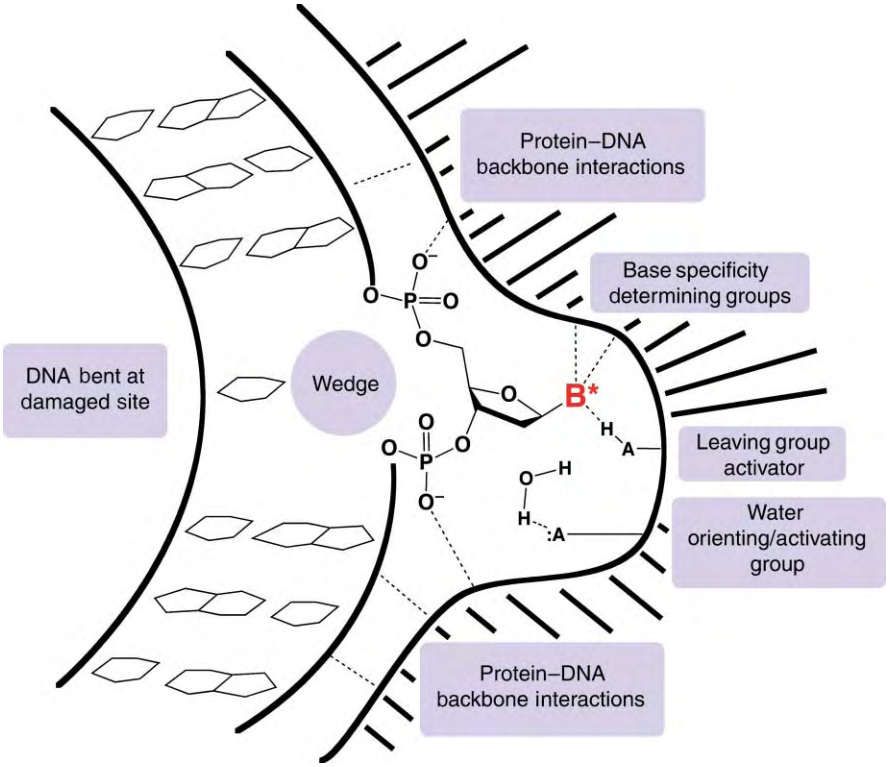



**FIGURE 1** Schematic representation of base flipping and other common interactions of DNA glycosylases with DNA. B*, damaged base; (---), hydrogen bond.

Base flipping plays an important role in facilitating base recognition and glycosidic bond cleavage. Extrusion of the base juxtaposes its unique functional groups with complementary groups in the enzyme active site. These specific interactions promote the recognition of the damaged base and largely exclude binding of undamaged bases. Differences in the amino acid residues of the base binding pocket among DNA glycosylases, in part, account for differences in their substrate specificity and catalytic mechanisms (see later discussion). Base flipping also exposes the anomeric carbon (the deoxyribose carbon that is attached to the base) to attack by water in the enzyme active site. Thus, base flipping enables DNA glycosylases to couple damaged base recognition to enzyme catalysis.

## Catalytic Strategies for Glycosidic Bond Hydrolysis

At neutral pH and ambient temperature, the glycosidic bond of a deoxynucleotide is a very stable linkage toward hydrolysis ($t_{1/2} = 109$ years for thymidine; $t_{1/2} = 3.9$ years for deoxyadenosine). The stability of this bond stems in part from the poor leaving-group ability of the electron-rich nucleobase, the poor nucleophilicity of water, and the poor electrophilicity of the anomeric carbon. DNA glycosylases have evolved remarkably similar strategies to overcome these chemical problems and remove these lesions rapidly enough to preserve the integrity of the genomic information.

### Making the Cognate Nucleobase a Better Leaving Group

Most neutral deoxynucleotide bases are poor leaving groups because they cannot effectively stabilize the developing negative charge that forms on the base during the reaction. Some DNA glycosylases stabilize this negative charge by donating specific hydrogen bonds from active-site residues to acceptors on the base (Figure 1). This type of catalysis has been most clearly shown for UDG, in which an unusually strong hydrogen bond from a histidine residue in the enzyme active site stabilizes the uracil anion leaving group. In other cases, leaving group departure may be facilitated by the full transfer of a proton from an active site donor to a proton-accepting atom on the nucleobase, as has been suggested for MutY.

*N*-alkylated purines (i.e., 3-methyladenine and 7-methylguanine) are unique in that they are positively charged and electron-deficient; therefore, they are excellent leaving groups in the absence of any enzymatic activation. Accordingly, DNA glycosylases use different types of catalytic interventions to affect the hydrolysis of these labile bases. For example, the cationic base binding site of AlkA lacks the constellation of highly specific hydrogen-bonding groups that line the active sites of DNA glycosylases that recognize neutral damaged bases. Instead, its active site is rich in aromatic amino acids, such as tryptophan and tyrosine, that allow AlkA to bind purine bases of varying shape and account for the promiscuous activity of this enzyme. The unique chemical character of the AlkA active site may facilitate damaged-base recognition by forming favorable stacking interactions between the cationic *N*-alkylpurine and the aromatic side chains. Alternatively, positioning the cationic damaged base in a hydrophobic active site may serve to lower the activation barrier by electrostatic destabilization of the charged ground state. These key questions are still under active investigation.

### The Nature of the Transition State

The rupture of the glycosidic bond in DNA can proceed through two limiting routes (Figure 2). In the first route (Figure 2A), the attack of water is coincident with departure of the leaving base, resulting in a concerted transition state. In a concerted reaction, the anomeric carbon is relatively electron rich, and little negative charge has built up on the leaving nucleobase. In the second route (Figure 2B), the bond between the anomeric carbon and the nucleobase is completely broken before the new bond with water forms, and the reaction proceeds via a stepwise mechanism with a discrete intermediate. Thus, in a stepwise reaction, the anomeric carbon is electron poor and has a significant positive charge, and the bonding electrons have migrated fully onto the leaving-group nucleobase.

Although comprehensive mechanistic studies for most DNA glycosylases have not been performed, it appears that these enzymes may catalyze their reactions using mechanisms in which the glycosidic bond is mostly broken and the new bond between water and the deoxyribose is only partially formed. In support of these electronic features, custom oligonucleotides containing a positively charged deoxyribose analogue that mimics the electron-deficient anomeric carbon at the transition state are potent inhibitors of several DNA glycosylases, including UDG and AlkA (Figure 2C). Such inhibitors of DNA glycosylases may someday find use in increasing the efficacy of anticancer agents that modify the bases of DNA by disabling the repair pathways that reverse the effects of these chemotherapeutics.

One way in which DNA glycosylases stabilize stepwise reactions is by surrounding the positively charged deoxyribose with negatively charged groups. For example, UDG engulfs the cationic deoxyribose intermediate with a negatively charged aspartate residue, DNA phosphodiester groups, and the uracil anion leaving group.

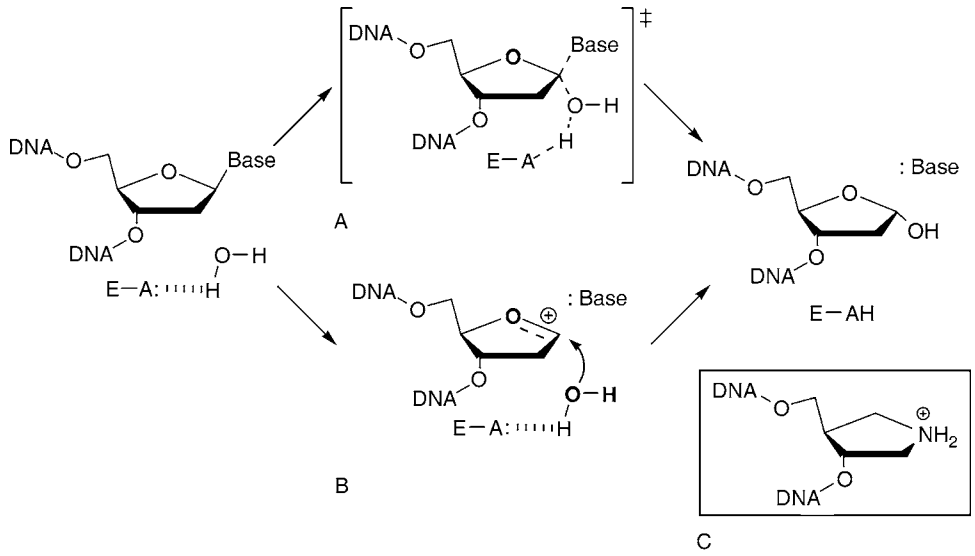



**FIGURE 2** Two limiting reaction pathways for DNA glycosylases. (A) Concerted pathway; (B) stepwise pathway; (C) DNA glycosylase inhibitor based on the cationic deoxyribose intermediate of the stepwise reaction.

### Helping Water Attack the Anomeric Carbon

Most DNA glycosylases possess a polar amino acid near the anomeric carbon of the target deoxyribose that can accept a hydrogen bond or proton from the attacking water (i.e., aspartate, glutamate, or asparagine) (Figure 1). Depending on the mechanism, this group may simply orient the water close to the anomeric carbon or, alternatively, may actively facilitate its attack by deprotonation. In concerted reactions, when there is significant bond formation between the attacking water and the anomeric carbon in the transition state, the activation barrier is lessened by partial or full deprotonation of the water. For stepwise reactions, the water needs only to be oriented close to the positively charged anomeric carbon because such species are highly reactive even with poor nucleophiles such as water (Figure 2B).

## See Also the Following Article

DNA Base Excision Repair

## Glossary

**abasic site** A deoxyribose residue in DNA that lacks a base.

**base flipping** The process by which an enzyme extrudes a base from within the DNA double helix into an extrahelical position inside the active site of the enzyme.

**DNA glycosylase** A family of functionally related enzymes that catalyze the hydrolytic cleavage of a damaged base from DNA.

**glycosidic bond** The bond connecting the anomeric carbon of the deoxyribose to the nitrogen on the purine or pyrimidine base.

**transition state** The highest potential energy species in the overall transformation of reactants to products. The difference in energy between the reactants and the transition state (the activation barrier) determines the rate of reaction.

## Further Reading

Hollis, T., Ichikawa, Y., and Ellenberger, T. (2000). DNA bending and a flip-out mechanism for base excision by the helix-hairpin-helix DNA glycoyslase, *Escherichia coli* AlkA. *EMBO J.* **19**, 758–766.

Jiang, Y. L., Drohat, A. C., Ichikawa, Y., and Stivers, J. T. (2002). Probing the limits of electrostatic catalysis by uracil DNA glycosylase using transition state mimicry and mutagenesis. *J. Biol. Chem.* **277**, 15385–15392.

Pearl, L. H. (2000). Structure and function in the uracil–DNA glycosylase superfamily. *Mutat. Res.* **460**, 165–181.

Stivers, J. T., and Drohat, A. C. (2001). Uracil DNA glycosylase: Insights from a master catalyst. *Arch. Biochem. Biophys.* **396**, 1–9.

Stivers, J. T., and Jiang, Y. L. (2003). A mechanistic perspective on the chemistry of DNA repair glycosylases. *Chem. Rev.* **103**, 2729–2759.

## Biography

Daniel J. Krosky is currently a graduate student at the Johns Hopkins University School of Medicine, studying the energetics of base flipping by uracil DNA glycosylase. Prior to attending Johns Hopkins University, he was a Senior Research Associate at AstraZeneca R&D, Boston, developing novel antibacterial agents.

James T. Stivers is an Associate Professor in the Department of Pharmacology and Molecular Sciences at Johns Hopkins Medical School. His research focuses on understanding the nature of enzyme catalysis and inhibition for a number of enzymes involved in DNA repair and recombination. He obtained his Ph.D. in biochemistry from Johns Hopkins University in 1992. He received postdoctoral training in heteronuclear NMR and enzymology in the laboratory of Professor Albert Mildvan at Johns Hopkins Medical School.

# DNA Helicases: Dimeric Enzyme Action

**Timothy M. Lohman**
*Washington University School of Medicine, St. Louis, Missouri, USA*

**DNA helicases are a class of motor proteins that function to generate the transient single-stranded DNA required as intermediates in DNA and RNA metabolism. These enzymes couple the energy obtained from the binding and hydrolysis of nucleoside 5′-triphosphates (NTP) to perform the work of DNA duplex unwinding (strand separation) and translocation of the helicase along the linear DNA filament. Helicases function in a variety of processes including DNA replication, DNA repair, recombination, and bacterial conjugation, and are components of eukaryotic transcription complexes. Mutations in enzymes with helicase activity result in a variety of human genetic diseases. This article focuses on the *Escherichia coli* Rep and UvrD helicases, both members of the SF1 helicase superfamily.**

## Function

The *Escherichia coli* Rep helicase is involved in replication, most likely playing a role in replication restart. Rep was first identified as being required for replication of bacteriophage $\phi$X174. Rep in complex with the phage $\phi$X174 gene A protein is highly processive, being able to unwind the entire genome ($>$6000 bp). However, in the absence of this accessory protein, Rep unwinds DNA with significantly lower processivity. *E. coli* UvrD, also known as helicase II, is involved in nucleotide excision repair and methyl-directed mismatch repair of DNA, as well as in plasmid replication. In the absence of accessory proteins, the unwinding processivity of UvrD is also relatively low. However, UvrD must be able to unwind at least 1000 bp processively during methyl-directed mismatch repair. Therefore, the processivity of each of these helicases can be increased through interactions with accessory proteins.

Most DNA helicases show a preference for unwinding duplex DNA possessing a ss-DNA flanking region or "tail" *in vitro*, and generally display a defined "polarity of unwinding" with respect to the backbone polarity of the ss-DNA tail that flanks the duplex DNA. Helicases that initiate unwinding more efficiently on DNA substrates with a 3′-ss-DNA tail are referred to as "3′–5′ helicases," whereas those that prefer DNA substrates possessing a 5′-ss DNA tail are referred to as "5′–3′ helicases." The *E. coli* Rep, UvrD and *B. stearothermophilus* PcrA helicases are 3′–5′ helicases, whereas the phage T4 Dda protein is a 5′–3′ helicase. These results suggest that a 3′–5′ helicase translocates in that direction along ss-DNA and this appears to be the case. At high-protein concentrations, *E. coli* UvrD can also initiate DNA unwinding at a nick *in vitro*, which is the biologically important site for initiation of unwinding in its roles in methyl-directed mismatch repair and excision repair.

Helicases are allosteric enzymes, many of which function as oligomeric assemblies. The class of hexameric DNA helicases exemplifies such oligomerization. The general importance of oligomerization for the function of SF1 helicases is less clear and is the subject of current study. Although there is evidence that a monomer of the phage T4 Dda helicase, an SF1 helicase, displays limited helicase activity *in vitro*, monomers of *E. coli* Rep and UvrD do not display helicase activity *in vitro*, but must dimerize in order to unwind DNA *in vitro*. The role of this dimerization may be to activate the enzyme and to provide the functional enzyme with multiple DNA-binding sites that are important for increasing the processivity of DNA unwinding.

## Structural Features of SF1 DNA Helicases

### Primary Structures

Helicases have been classified into families and superfamilies (SF1, SF2, SF3, F4, and F5) based on conserved amino acid sequence patterns. The SF1 superfamily (containing *E. coli* Rep and UvrD) is defined by seven conserved regions of primary structure, referred to as "helicase motifs." The only regions of sequence similarity that are shared uniformly among all of the helicase families are motifs I and II, which correspond to the

 © 2004, Elsevier Inc. All Rights Reserved. 

"Walker A" and "B" boxes that form part of the nucleoside-5′-triphosphate-binding site. The presence of these two motifs is necessary but not sufficient for a protein to have helicase activity. The remaining conserved regions of the SF1 helicases are involved in binding of nucleoside-5′-triphosphate and/or DNA.

## Crystal Structures of SF1 Helicases

Crystal structures have been solved for three SF1 DNA helicases, *B. stearothermophilus* PcrA, *E. coli* Rep, and *E. coli* UvrD. The apo form of PcrA as well as PcrA in complex with ADP, was solved at 2.5 and 2.9Å resolution, respectively. Structures of *E. coli* Rep bound to ss-DNA ($dT(pT)_{15}$) and Rep bound to ss-DNA and ADP were solved at 3.0 and 3.2Å, respectively. Structures of PcrA in complex with a 3′-ss-ds DNA junction, both in the presence and absence of the nonhydrolyzable ATP analogue, AMPPNP, were solved at 3.3 and 2.9Å, respectively.

The Rep (673 amino acids), PcrA (675 amino acids), and UvrD (720 amino acids) proteins are structurally homologous; a structure of the Rep-ss-DNA-ADP ternary complex is shown schematically in Figure 1. These SF1 proteins consist of two domains (1 and 2), separated by a cleft; domains 1 and 2 are further composed of subdomains 1A, 2A, 1B, and 2B (Figures 1 and 2). Subdomains 1A and 2A are composed of central

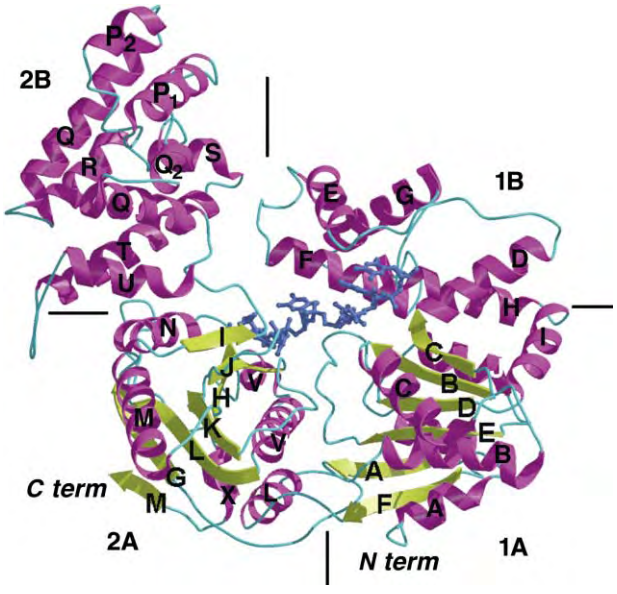



**FIGURE 1** Structure of the *E. coli* Rep monomer bound to a single-stranded DNA (6 nucleotides shown in blue). Subdomains 1A, 1B, 2A, and 2B are indicated. The 3′ end of the ss-DNA resides near domain 1. Reprinted from Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997). Major domain swiveling revealed by the crystal structures of complexes of *E. coli* Rep helicase bound to single-stranded DNA and ADP. *Cell* **90**, 635–647.

β-sheets flanked by α-helical regions and are homologous to each other as well as to the nucleoside-5′-triphosphate-binding domain of the *E. coli* RecA protein. Subdomains 1A and 2A both contain large insertions which form the α-helical 1B and 2B domains.

The crystal structures of *E. coli* Rep and *B. stearothermophilus* PcrA in conjunction with mutagenesis studies on several helicases indicate that amino acid residues in the seven conserved SF1 "helicase motifs," I–VI and IA are involved in either nucleotide and/or DNA binding. As shown in Figure 2, motifs I, IA, II, and III are contained within domain 1A, motifs V and VI are contained within domain 2A, whereas domain IV is found at the cleft formed between domains 1A and 2A. In general, ADP interacts with residues in motifs I and IV, whereas ATP interacts with residues within or near all of the motifs, with the apparent exception of motif IA.

Rep binds ss-DNA with a defined orientation with respect to the polarity of the sugar-phosphate backbone. In the crystal structures, the 3′–5′ direction runs from domain 1 to domain 2 across the cleft between the two domains, with the 3′ end of the ss-DNA located in a groove formed between subdomains 1A and 1B.

Several of the "helicase motifs" appear to be involved in the transmission of information between the ATP and ss-DNA-binding sites within a Rep monomer. Motif V residues are also likely to be involved in both ss-DNA binding as well as ATP binding through interactions with the γ-phosphate. Motif VI interacts directly with the γ-phosphate of ATP, as well as with motif IV. The above summary considers only interactions that occur within a single monomer or subunit. However, since oligomerization of both Rep and UvrD is functionally important for helicase activity *in vitro*, important allosteric interactions also occur between subunits of a Rep dimer.

## "Open" and "Closed" Conformations

The asymmetric unit of the Rep-ssDNA crystal structure shows two molecules of Rep bound to a single molecule of $dT(pT)_{15}$. The conformations of the two Rep monomers are strikingly different with respect to the orientation of the 2B subdomain relative to the other three subdomains. These two conformations, referred to as "closed" and "open," respectively, differ by a ~130° rotation of the 2B subdomain about a hinge region connecting it to the 2A subdomain. It has been speculated that the 2B subdomain might be part of the interface between subunits within the Rep dimer, while others have suggested that the 2B subdomain forms part of the duplex DNA-binding site in the closed form of the PcrA monomer and plays a role in

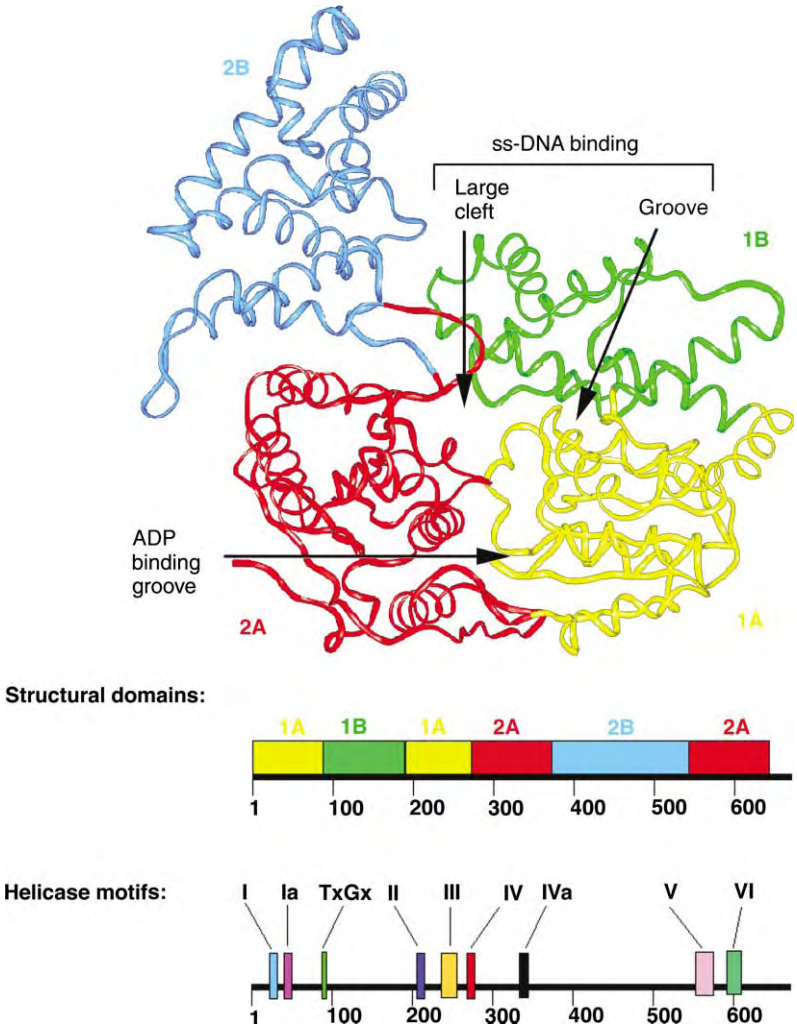



**FIGURE 2** The domain structure of *E. coli* Rep monomer. The positions of the subdomains and the helicase motifs within the primary structure of Rep are also shown. The color scheme for the individual motifs corresponds to that shown in Figure 1. Modified from Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997). Major domain swiveling revealed by the crystal structures of complexes of *E. coli* Rep helicase bound to single-stranded DNA and ADP. *Cell* **90**, 635–647.

duplex DNA stabilization. The essential nature of the 2B subdomain in Rep was tested by making a *rep* gene construct in which the coding region for the entire 2B domain was deleted and replaced by three glycines. The resulting RepΔ2B protein retains helicase activity *in vitro* and can also support $\phi$X174 phage replication *in vivo*, thus ruling out models invoking an essential role for the 2B domain in the helicase activity of Rep. In fact, a monomer of the RepΔ2B protein displays limited helicase activity *in vitro* in stark contrast to a wild-type Rep monomer which displays no helicase activity *in vitro*. This indicates that individual Rep monomers do possess all that is needed to unwind duplex DNA. However, the 2B subdomain appears to be inhibiting helicase activity of the monomer, and thus may have a regulatory function. This inhibition is relieved upon formation of a Rep or UvrD dimer.

## Single-Stranded DNA Translocation by Monomers of SF1 Helicases

Recent studies have demonstrated that monomers of *B. stearothermophilus* PcrA can indeed translocate with biased directionality (3′–5′) along ss-DNA in an ATP-dependent reaction. In fact, monomers of *E. coli* Rep and UvrD, which are structural homologues of PcrA, also have been shown to be able to translocate along ss-DNA with biased (3′–5′) directionality. However, since monomers of Rep and UvrD are unable to unwind duplex DNA *in vitro*, it is clear that ss-DNA translocation alone is insufficient for DNA helicase activity for these helicases, and that dimerization is required.

## DNA Unwinding by *E. coli* Rep and UvrD Helicases

### Rep and UvrD Monomers are Unable to Unwind Duplex DNA and Protein Oligomerization is Required for Helicase Activity *in vitro*

Single-turnover DNA-unwinding studies indicate that Rep monomers are unable to unwind DNA *in vitro* and that Rep oligomerization is required for initiation of DNA helicase activity *in vitro*. Single-molecule fluorescence techniques have shown that a monomer of Rep uses ATP hydrolysis to translocate toward the ss/dsDNA junction, but then displays futile ATP-dependent conformational fluctuations, followed by dissociation of the monomer. DNA unwinding is initiated only if a functional oligomeric helicase is formed. Significantly, partial dissociation of the oligomeric helicase during unwinding leaves an inactive Rep monomer, resulting in a stalled complex. This stalled complex can be resolved in two ways. Dissociation of the remaining bound monomer leads to rewinding of the DNA duplex; however, re-initiation of unwinding can occur upon re-formation of the functional helicase by binding of additional Rep protein. These results show that a Rep monomer is unable to sustain DNA unwinding after the active Rep oligomeric complex disassembles. This also suggests that the low unwinding processivity observed for Rep DNA unwinding *in vitro* may be due to the relative instability of the functional dimer.

Initiation of DNA unwinding *in vitro* also requires a dimeric UvrD complex in which one subunit is bound to the ss/ds-DNA junction, while the second subunit is bound to the 3′ ss-DNA tail. Since the assay used in these UvrD studies relies on complete unwinding of an 18 base pair duplex, these results cannot exclude the possibility that monomers might unwind a short stretch of DNA duplex; however, processive unwinding of an 18 bp duplex requires UvrD oligomerization. Therefore, simple unidirectional translocation of a UvrD or Rep monomer along ss-DNA is not sufficient for helicase activity, and thus do not support "passive" models of DNA unwinding which assume that a translocating enzyme can unwind a duplex by simply taking advantage of the thermal fluctuations at the ss–ds DNA junction that result in transient fraying of the duplex end.

### Kinetic Estimate of the DNA Unwinding "Step-Size" for *E. coli* UvrD Helicase

A kinetic approach indicates that UvrD unwinds 4–5 bp in each step (called the kinetic step size). This indicates that a rate-limiting step is repeated during the unwinding cycle and the unwinding "step-size" of 4–5 bp represents the number of base pairs unwound between two successive rate-limiting steps. It is not yet known how this kinetic step-size is related to a mechanical step size. Similar experiments performed with *E. coli* RecBCD helicase yield a step size of $3.9 \pm 1.3$ bp. A value of $\sim 6$ bp per step has also been estimated for the vaccinia NPH-II helicase. The similarities among these step sizes suggest mechanistic similarities, although this remains to be determined.

## Mechanisms for DNA Unwinding and Translocation by SF1 Helicases

Whereas it seems likely that SF1 helicases may share some features of their unwinding mechanisms, it is still too early to conclude whether a single mechanism applies to all SF1 helicases. In fact, there are clear differences among some of the SF1 helicases studied to date. Although *E. coli* Rep and UvrD must oligomerize to function as helicases *in vitro*, a monomer of the phage T4 Dda helicase possesses limited unwinding activity *in vitro*. It is therefore possible that some SF1 helicases function as monomers to unwind short stretches of duplex nucleic acids, while oligomerization may be necessary to activate some SF1 helicases or to enhance their processivity. Of course, interaction of a helicase with an accessory protein may also modify the need for the helicase to oligomerize by providing a second DNA-binding site.

Most proposed mechanisms for DNA unwinding and helicase translocation assume the functional helicase to possess at least two DNA-binding sites. Differences in current models for DNA unwinding center on three aspects: (1) whether translocation of the helicase along ss-DNA is sufficient for helicase activity or whether the helicase also interacts directly with the duplex DNA; (2) whether the two DNA-binding sites are contained within a single polypeptide; and (3) whether the same DNA-binding site remains as the lead site (inch-worm model) or whether multiple sites alternate as the lead subunit ("rolling" or "hand-over-hand" models).

### Active Versus Passive Mechanisms of DNA Unwinding

One operational distinction among DNA unwinding models is whether the helicase participates directly in destabilizing the duplex DNA (active mechanism), or whether the helicase interacts solely with the ss-DNA and waits for transient fluctuations in the duplex to form a ss-DNA region (end fraying) onto which the helicase can then translocate (passive mechanism).

"Active" mechanisms would generally involve direct binding of the helicase to the duplex DNA (or the duplex–ss DNA junction), in addition to the ss-DNA, although a "torsional" mechanism is possible in which binding of the helicase to both single strands might unwind the duplex without direct duplex interactions.

Tests of a "passive" mechanism of unwinding have been made for *E. coli* Rep and UvrD helicases using non-natural DNA substrates in which a region of the ss-DNA adjacent to the duplex was reversed in its backbone polarity or replaced by polyethylene glycol. The segments of polyethylene glycol or reversed polarity ss–DNA would prevent a helicase from translocating up to the ss–ds DNA junction, thus a helicase operating by a "passive" mechanism would not be expected to unwind such DNA substrates. However, Rep and UvrD can unwind such DNA molecules, ruling out a passive mechanism for these helicases.

## Dimeric, Subunit Switching Models

### Active, "Rolling" or "Hand-Over-Hand" Models for a Dimeric Helicase

An "active, rolling" mechanism (Figure 3A) has been proposed for how a Rep dimer might unwind DNA and translocate along the DNA filament. In this model, the individual subunits alternate as the lead subunit, which binds to duplex DNA, followed by unwinding. However, all of the evidence upon which this model was based is also consistent with a "dimeric inch-worm" model shown schematically in Figure 3B.

### Dimeric Inch-Worm Models

The only difference between the "rolling" and "dimeric, inch-worm" models is that the same subunit remains as the lead subunit in the "dimeric inch-worm" model. In fact, recent evidence with both Rep and UvrD favors a "dimeric, inch-worm" model rather than a "rolling" or "hand-over-hand" model. Based on the fact that monomers of Rep and UvrD are able to translocate along ss-DNA with biased (3′–5′) directionality, it seems most likely that in a "dimeric inch-worm" model, the trailing subunit of the dimer might maintain continuous contact with the 3′ ss-DNA, possibly providing the motor component of the helicase, whereas the leading subunit would interact with the ss–ds DNA junction. However, such details are still speculative and need to be tested.

## Monomeric Inch-Worm Models

It has been proposed that that a PcrA monomer unwinds DNA by an inch-worm mechanism. Support for the monomeric inch-worm model proposed for PcrA comes from the fact that PcrA monomers are able to translocate along ss-DNA with biased (3′–5′) directionality. However, both Rep and UvrD monomers also possess this ability, yet cannot unwind DNA *in vitro*.

## Summary

It is now clear that monomers of SF1 helicases are able to translocate along ss-DNA with biased directionality in an ATP-dependent reaction. These monomers must use some sort of inch-worm mechanism for this translocation. However, monomers of *E. coli* Rep and UvrD are not able to initiate DNA unwinding *in vitro*, despite the fact that these monomers can translocate along ss-DNA with directional bias. Therefore, the ability to translocate with unidirectional bias along ss-DNA is not sufficient for a protein to have helicase activity. In fact, to date, only the phage T4 Dda and a deletion mutant of *E. coli* Rep (RepΔ2B) have been shown to unwind DNA *in vitro* as monomers, and these do so with low processivity. The 2B subdomain of Rep is not required for its helicase activity *in vitro*; in fact, this domain inhibits the ability of a Rep monomer to unwind

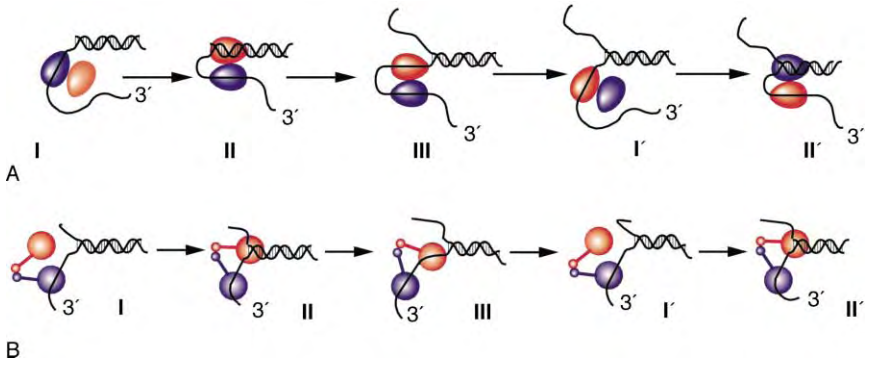



**FIGURE 3** Models for DNA unwinding and translocation by a dimeric helicase. (A) Active, "rolling" or "hand-over-hand" model, and (B) dimeric "inch-worm" model.

DNA *in vitro*. Both *E. coli* Rep and UvrD are required to dimerize in order to initiate DNA unwinding *in vitro*, thus the inhibition of helicase activity by the 2B subdomain of Rep appears to be relieved through Rep oligomerization.

## See Also the Following Articles

DNA Helicases: Hexameric Enzyme Action • DNA Mismatch Repair in Bacteria • DNA Replication Fork, Bacterial • DNA Replication: Initiation in Bacteria • DNA Topoisomerases: Type III–RecQ Helicase Systems • recQ DNA Helicase Family in Genetic Stability

## Glossary

**helicase** An enzyme that utilizes nucleoside 5′-triphosphates to separate the two strands of the DNA double helix in order to form the single-stranded DNA intermediates that are required for DNA metabolism (i.e., replication, recombination, and repair).

**kinetic step size** The average number of base pairs unwound between successive, repeated rate-limiting steps in the DNA unwinding cycle.

**processivity** A measure of the average number of base pairs that can be unwound by a helicase before it dissociates from the DNA.

## Further Reading

Ellis, N. A. (1997). DNA helicases in inherited human disorders. *Curr. Opin. Genet. Dev.* **7**, 354–363.

Geider, K., and Hoffmann-Berling, H. (1981). Proteins controlling the helical structure of DNA. *Annu. Rev. Biochem.* **50**, 233–260.

Kornberg, A., and Baker, T. A. (1992). *DNA Replication*. W.H. Freeman, New York.

Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997). Major domain swiveling revealed by the crystal structures of complexes of *E. coli* Rep helicase bound to single-stranded DNA and ADP. *Cell* **90**, 635–647.

Lohman, T. M., and Bjornson, K. P. (1996). Mechanisms of helicase-catalyzed DNA unwinding. *Annu. Rev. Biochem.* **65**, 169–214.

Matson, S. W., and Kaiser-Rogers, K. A. (1990). DNA helicases. *Annu. Rev. Biochem.* **59**, 289–329.

Matson, S. W., Bean, D. W., and George, J. W. (1994). DNA helicases: Enzymes with essential roles in all aspects of DNA metabolism. *Bioessays* **16**, 13–22.

Modrich, P. (1987). DNA mismatch correction. *Annu. Rev. Biochem.* **56**, 435–466.

Modrich, P. (1994). Mismatch repair, genetic stability, and cancer. *Science* **266**, 1959–1960.

Patel, S. S., and Picha, K. M. (2000). Structure and function of hexameric helicases. *Annu. Rev. Biochem.* **69**, 651–697.

Soultanas, P., and Wigley, D. B. (2001). Unwinding the gordian knot of helicase action. *TIBS* **26**, 47–54.

## Biography

Timothy M. Lohman is the Marvin A. Brennecke Professor of Biological Chemistry and a member of the Department of Biochemistry and Molecular Biophysics at Washington University School of Medicine, St. Louis, MO. His principal research interests are in the study of energetics and kinetic mechanisms of protein–DNA interactions, focusing on helicases and SSB proteins. He holds a Ph.D. in Physical Chemistry from the University of Wisconsin, Madison and did postdoctoral research at the University of California, San Diego and the University of Oregon.

# DNA Helicases: Hexameric Enzyme Action

**Smita S. Patel**
*Robert Wood Johnson Medical School, Piscataway, New Jersey, USA*

**Helicases are molecular motor proteins that use the energy of nucleoside triphosphate (NTP) hydrolysis to unidirectionally translocate along nucleic acid while separating the double-strand (ds) DNA strands, removing secondary structures in RNA, and displacing proteins bound to nucleic acids. Helicases are integral parts of the cellular machinery involved in DNA and RNA metabolic processes. Thus, it is not surprising that helicases and helicase-related proteins constitute more than 2% of the eukaryotic genome. Mutations in human genes coding for helicases result in several diseases that result in cancer and premature aging. Helicases also play a key role in viral life cycles such as viral genome replication and genome packaging/unpackaging. Viral helicases are therefore attractive targets for antiviral therapy. The topic of helicase structure, function, and mechanisms has been widely discussed in the literature. Oligomeric state divides helicases into two groups: ring (hexameric) helicases and all other. In this article, we start by discussing general mechanisms of translocation and strand separation of nucleic acid by helicases, which is followed by a discussion of the mechanisms of ring helicases.**

## Nucleic Acid Strand Separation Activity of Helicases

A long stretch of ds DNA or RNA is unwound by the helicase in a step-wise manner as shown in Figure 1. In each step, the helicase translocates and separates the duplex strands.

Most helicases require a stretch of single-stranded (ss) nucleic acid of a specific polarity adjacent to the duplex region to initiate strand separation. Helicases that require a 3′ ss tail to initiate strand separation are assumed to translocate in the 3′ to 5′ direction, and *vice versa*. Some helicases including hexameric ring helicases like T7 phage gp4 helicase, T4 phage gp41 helicase, and bacterial DnaB helicase require both 5′ and 3′ non-complementary tails to initiate strand separation, in which case it is difficult to determine the directionality of translocation using this assay.

Nucleic acid strand separation is commonly measured using short duplexes that contain a labeled strand of nucleic acid. Helicase is mixed with the substrate and MgNTP is added to initiate the reaction. This is a discontinuous assay, and after various reaction times, sodium dodecylsulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) are added to stop the reaction. It is assumed that partially separated strands reanneal and only the completely separated strands are observed as products by native polyacrylamide gel electrophoresis. Alternatively, strand separation is measured in real time by using fluorescently labeled nucleic acids. This can be measured in ensemble reactions by the stopped flow method by monitoring fluorescence change or loss of fluorescence resonance energy transfer (FRET) as the strands are separated or by using FRET in single molecule experiments.

Multiple enzymatic activities are required for the helicase function, including NTPase, translocation, and strand separation. All helicases hydrolyze NTP, commonly ATP, both in the absence and in the presence of nucleic acid. The NTPase activity in the absence of nucleic acid is low and is stimulated many fold in the presence of nucleic acid. The stimulated NTPase activity is coupled to strand separation and unidirectional translocation of the helicase along nucleic acid. It is believed that as the helicase cycles through different NTP ligation states, the reactions of NTP binding, hydrolysis and product release induce conformational changes in the helicase's nucleic acid binding site. These conformational changes alter the affinity of the helicase for the substrate and/or to perform a power stroke to somehow drive unidirectional translocation and strand separation.

The helicase activity can be broadly divided into two reactions: unidirectional translocation and strand separation, both of which are fueled by the NTPase activity of the helicase. Several studies indicate that helicases translocate unidirectionally along ss DNA and that unidirectional translocation does not require the presence of the ss/ds DNA junction. Thus, it is likely that unidirectional translocation along nucleic acid is

 © 2004, Elsevier Inc. All Rights Reserved. 

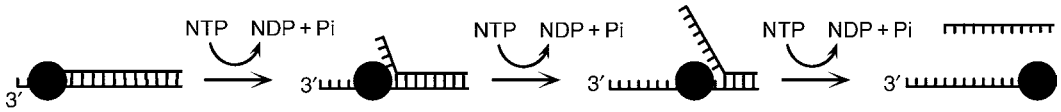



**FIGURE 1** Helicase separating the strands of nucleic acid in the 3′–5′direction. The helicase (black circle) initiates strand separation by binding to the 3′-tail, translocating along a strand of the ss/ds nucleic acid substrate, and separating the strands of the substrate as it hydrolyzes NTP to NDP and phosphate (Pi).

the basic activity of helicases that also facilitates the strand separation process. A number of mechanisms of strand separation and NTPase-coupled translocation have been proposed from studies of different helicases. Some of the differences in the proposed mechanisms result from the different biochemical properties of the helicases, such as the protein oligomeric state, the interactions with ss versus ds substrates, and the effect of the nucleotide ligation state of the helicase on its substrate binding properties. Thus, it might appear that there are several ways to separate the strands of nucleic acid; although, it is likely that as detailed studies are carried out, a general mechanism of translocation and strand separation by helicases will emerge.

## Mechanisms of Unidirectional Translocation

### Stepping Model

It is commonly believed that translocation requires at least two nucleic acid binding sites that independently bind and release nucleic acid as the helicase goes through the NTPase cycle. Such stepping models have been discussed widely and proposed for helicases such as PcrA, Rep, and UvrD. The two nucleic acid binding sites move with respect to each other in response to the conformational changes induced by NTP binding, hydrolysis, and product release at the NTP binding site of the helicase. In an oligomeric helicase, the NTPase activity of each subunit controls the conformation of the nucleic acid binding site; thus, coordinated NTPase activity leads to coordinated binding and release of the helicase from nucleic acid. Several helicases including PcrA, HCV helicase, and T4 DdaA are active as monomers. A monomeric helicase with one nucleic binding site cannot use the stepping mechanism described above. The monomer of PcrA has been proposed to contain two DNA binding sites that coordinate DNA binding and release. In this case, the two DNA binding sites on a monomer are controlled by one nucleotide binding site.

### Brownian Motor Model

An alternative model of translocation integrates Brownian motion into a molecular motor. Such a Brownian motor mechanism has been proposed for motors such as kinesin, myosin, and ion pumps. A Brownian motor uses thermal fluctuations from its surroundings and the NTPase reaction to achieve unidirectional movement. Brownian motor model can be applied to monomeric helicases with one nucleic acid binding site, although it predicts limited processivity of translocation and strand separation. The helicase holds on to the ss nucleic acid only via one binding site, and therefore it is more likely to dissociate during the weak binding sliding phase. A dimeric helicase with two and a hexameric helicase with six nucleic acid binding sites can use a Brownian motor mechanism and translocate with a greater efficiency. This is especially true if the helicase subunits coordinate their NTPase cycles and take turns binding and hydrolyzing NTP. In fact, a coordinated dimer or hexamer should appear as if it is stepping, because its subunits are taking turn moving forward.

Figure 2 shows a mechanism in which we suppose that in the absence of NTP the helicase is tightly bound to the nucleic acid and hence is in its lowest energy state unable to move along the substrate. When NTP binds to the helicase, it causes a conformational change in its nucleic acid binding site, resulting in a weakening of the nucleic acid binding affinity. In this weak binding state, the helicase's binding free energy is constant along the nucleic acid length (Figure 2, dotted line), which allows the helicase to slide along randomly in either direction influenced by thermal fluctuations, this is Brownian motion (Figure 2, position 4). It is important that the random movement of the helicase lasts only for a short time, and this is achieved by rapid NTP hydrolysis. After NTP hydrolysis, the tight rebinding to the nucleic acid is associated with a forward movement of the helicase along the direction of translocation. The forward movement of the helicase occurs along a decreasing energy slope and can be considered a power stroke. The forward movement does not require specific interactions with the ss/ds junction; hence, it can provide unidirectional movement along ss nucleic acid. If Brownian motion moves the helicase backward, a forward power stroke while rebinding to the nucleic acid will bring the helicase back to the same position it started, without net movement (position 3). The helicase molecules that diffuse in the forward direction (position 5) will end up one step forward from their original position upon tight

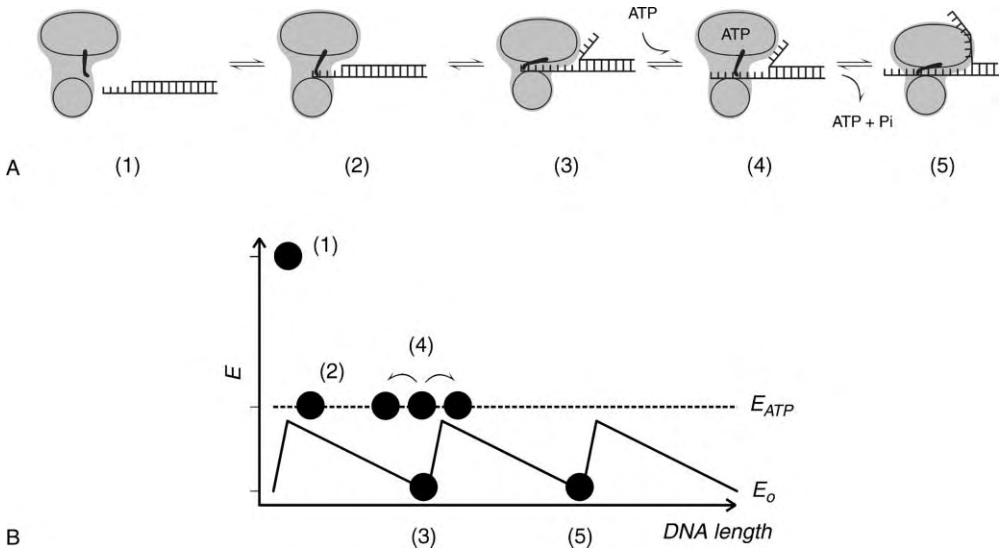



**FIGURE 2** Translocation of the helicase by a Brownian motor mechanism. (A) Transition of the helicase from a weakly nucleic-acid-bound state (helicase-ss nucleic acid-ATP state, (2)) to a tightly nucleic acid-bound state (helicase-ss nucleic acid state, (3)) is coupled to unidirectional movement. (B) Helicase binding free energy (E) along the length of ss nucleic acid. The helicase–nucleic acid complex has an asymmetric saw tooth profile along the length of ss nucleic acid, $E_o$ (solid line) in the nucleotide-free state and a flat energy profile in the ATP-bound state, $E_{ATP}$ (dotted line). The helicase position on ss nucleic acid is represented by gray circles: (1) free helicase; (2) helicase bound to ss nucleic acid weakly; (3) helicase bound to ss nucleic acid tightly; (4) helicase bound weakly to ss nucleic acid that can diffuse in either direction; (5) rebinding of the helicase from position (4) results in a net forward movement.

rebinding to the nucleic acid. A helicase can move forward even against a moderate external force (such as strand separation) as long as a significant fraction of the helicase can diffuse forward to position 5. Repeated binding and release of the helicase catalyzed by NTPase cycles results in a net unidirectional translocation of the helicase. This mechanism may be considered a combination of power stroke and Brownian ratchet.

The efficiency of a motor is defined as the fraction of productive steps per round of NTP hydrolysis. Several factors affect the efficiency of a Brownian motor. The greater the asymmetry of the saw tooth energy profile, the greater the efficiency of the motor. The steeper energy profile will increase the fraction of helicase molecules that reach position 5. The efficiency is also highly dependent on the rate of NTP hydrolysis, which determines the lifetime of the weakly bound state (Figure 2, position 4). If NTP hydrolysis is too fast, the lifetime of the weakly bound state will be too short and most molecules will end up in the same position they started with no net directed motion. If NTP hydrolysis is too slow, the molecules will have time to spread broadly in both directions with no net directed motion.

## Mechanisms of Nucleic Acid Strand Separation

Separating the strands of a duplex nucleic acid involves breaking the hydrogen bonds that hold the base pair (bp) together. At physiological temperature, nucleic acid bps open and close spontaneously. The rates of spontaneous bp opening as measured by imino proton exchange range from 30 $s^{-1}$ for internal bp to 1000 $s^{-1}$ for bp at the ss/ds junction. Individual bps open at a fast rate but the equilibrium for this reaction is toward bp formation. Therefore, bp closing is fast and it poses a kinetic barrier against the movement of helicase at the ss/ds nucleic acid junction.

At physiological temperatures, nucleic acid strand separation is a thermodynamically unfavorable process. In order to make the process thermodynamically favorable, the helicase must stabilize the transition state and/or stabilize the open bps. This can be accomplished either actively or passively. In an active mechanism, the helicase increases the rate of bp opening. If the helicase separates the nucleic acid strands by the passive mechanism, by definition, it does not change the rate of bp opening. A helicase can increase the rate of strand separation by lowering the transition state energy by binding to an intermediate such as a distorted duplex region or the ss/ds nucleic acid junction. Interaction of the helicase with ds nucleic acid has been viewed as an indication of an active mechanism of strand separation. Another way to actively disrupt bps is by unidirectional translocation or by a wedge mechanism. In this mechanism, the helicase translocates unidirectionally along ss nucleic acid and along its way it is able to capture base-pairs generated by thermal fluctuation. Helicases have been shown to move unidirectionally

along ss nucleic acid. Helicases have also been shown to displace proteins on their path and break the streptavidin–biotin linkage at the end of ssDNA without specifically interacting with them. Unidirectional translocation could be the driving force for strand separation and a basic property of helicases, making helicases similar to motor proteins that move on filaments.

## Step-Size of the Helicase

The physical step-size of a helicase is the number of bases it travels in a single cycle during unidirectional translocation; its measurement provides critical insights into the mechanism of translocation. Methods for measuring the physical step-size of a helicase directly have been reported, and there is a need to develop methods to quantitatively measure the stepping motion of the helicase. Kinetic step-size of a helicase can be measured by globally fitting the single turnover kinetics of strand separation of duplexes of various lengths. This method assumes that all species have identical stepping properties. In cases where multiple populations with different stepping rates exist, the kinetics will exaggerate the estimation of step-size. In the case of a helicase whose ATPase cycles are tightly coupled to movement, the step-size can also be obtained by measuring the coupling ratio, which is the number of bases the helicase can travel per NTP hydrolyzed.

## Hexameric Helicases

Hexameric ring helicases are found in all organisms including bacteriophage, viruses, bacteria, and eukaryotes. These helicases are often integral parts of protein complexes that catalyze nucleic acid replication, recombination, and transcription (Figure 3).

## Ring Structure

In most helicases, six identical polypeptides assemble into a hexameric ring. Eukaryotic minichromosome maintenance (MCM) helicases on the other hand assemble into a heterohexamer of Mcm2–7 proteins with a unique arrangement of the six subunits. The three-dimensional structure of ring helicases from various organisms has been characterized extensively by electron microscopy and image analysis. These studies show that the ring structure is conserved from bacteriophage to viruses and from prokaryotes to eukaryotes as shown in Figure 4. The six subunits are arranged in a toroidal shape. On an average, the outer diameter of the ring is approximately 12 nm and the diameter of the central channel is approximately

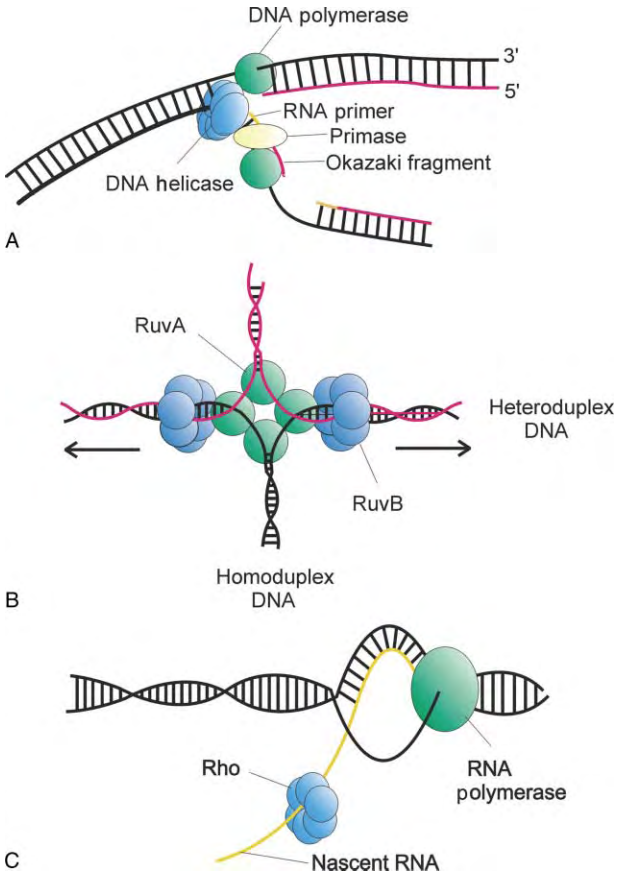



**FIGURE 3** Functions of ring helicases in (A) DNA replication, (B) recombination, and (C) transcription.

2 nm. The central channel is therefore wide enough to accommodate duplex DNA or RNA.

Crystal structures of ring helicases including RepA, RuvB, Rho, and fragments of T7 gp4 and SV40 Large T antigen provide atomic details of the subunit interface and the NTP binding site (Figure 5A). The crystallized RepA helicase ring in the absence of NTP has a sixfold symmetry, Rho ring was open, and the T7 gp4 ring with four NTPs bound has a twofold symmetry. The structure of the N-terminal fragment of archaea MCM protein reveals a double hexamer arrangement. The major subunit–subunit contact in RepA and T7 gp4 is between an N-terminal loop (in RepA) or the linker region between the helicase and primase domains (in T7 gp4) contacting helices between H1a and H2 of a neighboring subunit.

The structure of ring helicases shows that most of the helicase-conserved motifs including H1, H1a, H2, and H3 appear at the subunit interface, where they participate in NTP binding (Figure 5). Thus, NTP bound at the interface interacts with residues from adjacent subunits. A critical arginine (Arg 522 in T7 gp4) is found in hexameric helicases that come from a neighboring subunit and is within hydrogen bonding distance of the

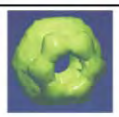
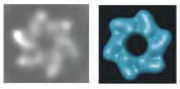
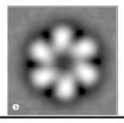
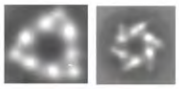
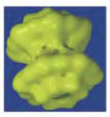
| Helicase | Mol. Wt. | Direction | Activities | Function | EM |
|---|---|---|---|---|---|
| *E. coli* DnaB | 52,390 | 5' to 3' | NTPase Helicase | DNA Replication |  |
| *E. coli* Bacteriophage T7 gp4 | 62,655 | 5' to 3' | Primase NTPase Helicase | DNA Replication |  |
| *E. coli* Bacteriophage T4 gp41 | 53,601 | 5' to 3' | NTPase Helicase | DNA Replication |  |
| Plasmid Encoded RSF1010 RepA | 29,909 | 5' to 3' | NTPase Helicase | DNA Replication |  |
| Simian virus large T antigen | 81,907 | 3' to 5' | NTPase Origin Binding Helicase | DNA Replication |  |
| Bovine papillomavirus E1 | 68,246 | 3' to 5' | NTPase Origin Binding Helicase | DNA Replication |  |
| *B. subtilis* Phage SPP1 gene 40 | 46,746 | 5' to 3' | NTPase Helicase | DNA Replication |  |
| *E. coli* RuvB | 37,174 | 5' to 3' | NTPase Branch Migration Helicase | DNA Recombination |  |
| *E. coli* Rho | 47,004 | 5' to 3' | NTPase Helicase | Transcription Termination |  |
| Human Bloom's syndrome helicase | 159,000 | 3' to 5' | NTPase Helicase | Unknown |  |

**FIGURE 4** Structures of ring helicases. Electron microscopy images of several ring helicases are shown.

gamma phosphate of NTP bound at the interface, as shown in Figure 5B. This residue is important for catalyzing NTP hydrolysis and is implicated in transducing conformational changes between subunits of the hexamer.

## Nucleic Acid Binding in the Central Channel

Detailed structural information is not available for the helicase nucleic acid binding site. Electron microscopy studies of ring helicases and biochemical studies indicate that nucleic acid binds in the central channel. Since the nucleic acid is confined within the central channel, its dissociation would require subunit–subunit disruption. This mode of nucleic acid binding is believed to confer processivity in translocation and strand separation activities of the hexameric helicases.

Processivity is the measure of the number of bases translocated or bp unwound before the helicase dissociates from the nucleic acid. Processivity, *P*, can be obtained from the measured stepping rate and the dissociation rate of the helicase. High processivity of translocation distinguishes the ring helicases from helicases such as *E. coli* UvrD, PcrA, and hepatitis C virus (HCV) helicases that do not assemble into rings.

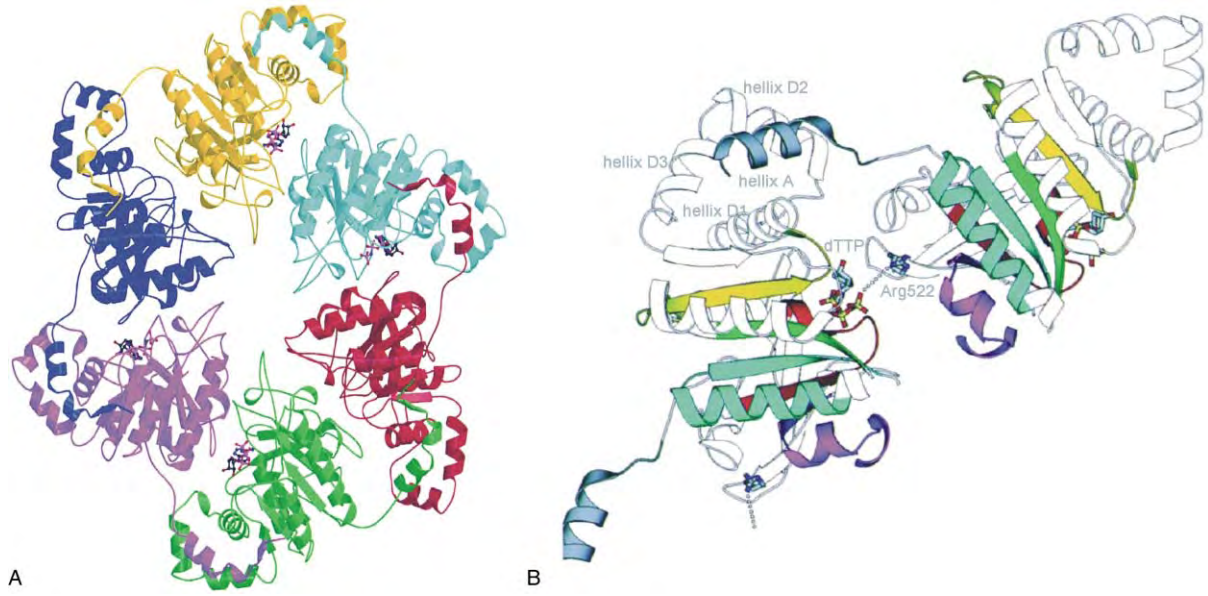



**FIGURE 5** Crystal structure of the helicase domain of T7 gp4 protein. (A) Model of a closed ring shaped hexamer with four adenosine 5′-($\beta,\gamma$-imido)triphosphates (ADPNP) bound at the subunit interfaces. (B) Arginine 522 (arginine-finger) contacting the nucleotide at the subunit interface.

Exceptions include RecBCD that may not encircle the DNA but shows a high processivity. The average processivity of ssDNA translocation by T7 gp4 hexamer on the other hand is $P = 0.99997$, a number close to one, which indicates that the probability of T7 helicase dissociating from ssDNA during translocation is very small. The processivity of T7 gp4 is only moderate because it is translocating and separating the strands of ds DNA, indicating that the association of T7 gp4 helicase with T7 DNA polymerase is necessary to achieve the high processivity observed during DNA replication. High processivity has been observed also for T4 gp41 and *E. coli* DnaB helicase in the replication complex.

How does the ring helicase bind nucleic acid in the central channel? The individual subunits can assemble around the nucleic acid, or the ring can open to accommodate the nucleic acid. Most helicases appear to use the ring-opening mechanism and they require a loader to efficiently bind nucleic acid. The loader is either part of the helicase or a separate protein or set of proteins that interact with the helicase. The primase site of T7 gp4 faces the outer surface of the ring and is proposed to play a role in loading the DNA into the central channel. Similarly, RNA wraps around the N-terminal domain of *E. coli* Rho protein, which plays a role in loading the RNA into the central channel. SV40 large T antigen and papilloma virus E1 contains origin binding domains that assist in DNA loading. In other ring helicases, accessory proteins are required to load the nucleic acid efficiently into the central channel. *E. coli* DnaB requires DnaC, T4 gp41 requires gp59, and MCM 2–7 helicase complex requires Cdc6 in addition to the single-stranded DNA binding proteins and origin recognition complex proteins to load at the replication origins.

## Mechanism and Role of NTP Hydrolysis

The role of NTPase activity in ring helicases is similar to that of other helicases, in that NTP acts as a molecular switch. For example, T7 gp4 binds ssDNA in the presence of deoxythymidine ($\beta$, $\gamma$, methylene)triphosphate (dTMP-PCP), a nonhydrolyzable analog of dTTP with a $K_d$ around 10 nM, and dTTP hydrolysis and Pi release leads to weakening of the interactions with ssDNA. Thus, dTTP binding and Pi release promote DNA binding and release events.

A negative cooperativity in nucleotide binding is observed in most hexameric helicases. A hexamer may bind only three nucleotides or show three high affinity and three low affinity nucleotide binding sites. T7 gp4 binds 3–4 nucleotides tightly in the presence or absence of Mg(II) ion. *E. coli* DnaB and Rho proteins have three high affinity and three low affinity nucleotide binding sites.

All six subunit interfaces of the hexamer can potentially bind and hydrolyze NTP. However, it has been proposed in T7 gp4 *E. coli* Rho as well as in MCM proteins that three subunits may have a regulatory role similar to that of the $F_1$-ATPase protein. Several possible mechanisms of NTP hydrolysis by ring helicases have

been discussed. The most appealing mechanism is sequential NTP hydrolysis by three or six of the hexameric subunits. Such a mechanism of NTP hydrolysis at three sites has been proposed based on pre-steady-state kinetic experiments in Rho protein with RNA and in T7 gp4 protein in the absence of ssDNA.

## Translocation along Single-Stranded and Double-Stranded Nucleic Acid

Hexameric helicases such as T7 gp4 and T4 gp41 have been shown to translocate unidirectionally along ssDNA. T4 gp41 was able to displace streptavidin complexed to biotin placed at the 3′-end but not the 5′-end, suggesting that it translocates in the 5′–3′ direction. Similarly, utilization of primase sites on ssDNA was used to show that T7 gp4 translocates in the 5′–3′ direction. The DNA-dependent NTPase rate of these helicases also shows a dependence on ssDNA length that can be interpreted as unidirectional translocation along DNA. The translocation rate on ssDNA was measured for T7 gp4 as 130 base/s (at 18°C), and while translocating, gp4 hydrolyzes dTTP at a rate of 50 $s^{-1}$. This indicates that the energy from hydrolysis of one dTTP on average leads to translocation of the helicase along three bases of ssDNA. T7 gp4 helicase therefore translocates with a minimal step-size of three bases along ssDNA. RuvB helicase binds and translocates on dsDNA, catalyzing Holliday junction migration. Recently, *E. coli* DnaB and T7 gp4 helicases have been shown to migrate on dsDNA, but the rate of translocation has not been determined.

Translocation along nucleic acid by hexameric helicases appears to require functional subunits. Mutant poisoning experiments show that substitution of one or two of the hexamer subunits with a mutant inhibits the helicase and nucleic acid-dependent NTPase activity. Pre-steady-state NTPase kinetic experiments have indicated that the subunits of the ring act in a cooperative manner. The crystal structure of T7 gp4 fragment reveals that three adjacent subunits are in different conformational state (Figure 5A). Based on the structure, it was proposed that the hexamer subunits interact sequentially with the DNA during translocation. The stepping mechanism consisting of sequential NTP hydrolysis by the hexameric subunits coupled to sequential binding and release of nucleic acid is an attractive mechanism, but it requires more experimental evidence. An alternative mechanism of translocation to that discussed above is the Brownian motor mechanism that cannot be ruled out for hexameric helicases.

## Binding and Strand Separation at the Fork Junction

Ring helicases encircle either the ss nucleic acid or the ds nucleic acid at the fork junction, as shown in Figure 3. Helicases involved in DNA replication are believed to encircle one of the ssDNA strands at the fork junction and to exclude the complementary strand from the central channel, as shown in Figure 3A. This mode of DNA binding minimizes immediate reannealing of the DNA strands after they are separated. The RuvB–RuvA complex involved in recombination (shown in Figure 3B) encircles dsDNA to drive Holliday junction movement. Recently, it was shown that when *E. coli* DnaB helicase and T7 gp4 helicase are presented with a substrate that contains only the 5′ ss DNA tail adjacent to the duplex they encircle and translocate along ds DNA and also drive movement of the Holliday junction. Transcription termination factor Rho is believed to translocate along RNA (shown in Figure 3C) to disrupt the transcription complex.

The kinetics of strand separation by helicase alone can be measured as described earlier using a small ds nucleic acid oligomer and by monitoring the release of ss nucleic acid. It was found with T7 gp4 that the rate of translocation along ssDNA is faster than the rate of ds DNA strand separation. Thus, T7 gp4 separates the ds DNA strands with a rate of about 15 bp/s. This rate depends on the concentration of NTP and temperature. Under the same experimental conditions, the rate of translocation along ssDNA is more than six times faster. Thus, dsDNA presents a barrier to the movement of helicase along DNA. The strand separation rate of helicases involved in replication also increases in the presence of the DNA polymerase. The replicative complex consisting of T7 gp4 helicase, gp2.5 protein, and T7 DNA polymerase catalyzes DNA replication at a rate greater than 200 bp/s at 30°C.

## Conclusion

A large number of helicases and helicase-like proteins have been identified by genome sequencing efforts. These proteins play essential roles in almost all nucleic acid metabolism processes, and many are known to be associated with human diseases such as cancer and premature aging. Interestingly, many viruses code for their own helicases, which make them unique targets for antiviral therapy. Understanding the structure and mechanisms of helicases is an important means to find the link between the complex human diseases and helicases and to aid in the drug discovery process. From the viewpoint of understanding how helicases work, there is still a lack of a general mechanism that would

explain nucleic acid strand separation and other functions performed by helicases at the molecular level. Despite all the differences in the properties and structures of helicases, it is possible that they function by similar mechanisms. It is likely that unidirectional translocation is a basic activity of all helicases responsible for nucleic acid strand separation and all other functions of helicases. This makes helicases similar to general motor proteins that translocate on lattices, and their mechanism of operation may apply to helicases as well. New methodologies such as analysis of single helicase molecules will allow direct observation of the helicase reaction, and more detailed information from these experiments will provide physical parameters that will reveal how helicases work.

## See Also the Following Articles

DNA Helicases: Dimeric Enzyme Action • DNA Polymerases: Kinetics and Mechanism • DNA Secondary Structure • recQ DNA Helicase Family in Genetic Stability

## Glossary

**Holliday junction** A four-stranded DNA structure that is an intermediate in DNA recombination.

**transition state** The highest energy species in a chemical reaction pathway.

## Further Reading

Astumian, R. D. (2001). Making molecules into motors. *Sci. Am.* **285**, 56–64.

Egelman, E. H., Yu, X., Wild, R., Hingorani, M. M., and Patel, S. S. (1995). Bacteriophage T7 helicase/primase proteins form rings around single-stranded DNA that suggest a general structure for hexameric helicases. *Proc. Natl Acad. Sci. USA* **92**, 3869–3873.

Hall, M. C., and Matson, S. W. (1999). Helicase motifs: The engine that powers DNA unwinding. *Mol. Microbiol.* **34**, 867–877.

Hingorani, M. M., Washington, M. T., Moore, K. C., and Patel, S. S. (1997). The dTTPase mechanism of T7 DNA helicase resembles the binding change mechanism of the F1-ATPase. *Proc. Natl Acad. Sci. USA* **94**, 5012–5017.

Kim, D. E., Narayan, M., and Patel, S. S. (2002). T7 DNA helicase: A molecular motor that processively and unidirectionally translocates along single-stranded DNA. *J. Mol. Biol.* **321**, 807–819.

Levin, M. K., and Patel, S. S. (2003). Helicases as molecular motors. In *Molecular Motors* (M. Schliwa, ed.) pp. 179–198. Wiley-VCH Verlag GmbH, Weinheim, Germany.

Lohman, T. M., and Bjornson, K. P. (1996). Mechanisms of helicase-catalyzed DNA unwinding. *Annu. Rev. Biochem.* **65**, 169–214.

Patel, S. S., and Picha, K. M. (2000). Structure and function of hexameric helicases. *Annu. Rev. Biochem.* **69**, 651–697.

Singleton, M. R., Sawaya, M. R., Ellenberger, T., and Wigley, D. B. (2000). Crystal structure of T7 gene 4 ring helicase indicates a mechanism for sequential hydrolysis of nucleotides. *Cell* **101**, 589–600.

Soultanas, P., and Wigley, D. B. (2001). Unwinding the 'Gordian knot' of helicase action. *Trends Biochem. Sci.* **26**, 47–54.

von Hippel, P. H., and Delagoutte, E. (2001). A general model for nucleic acid helicases and their "coupling" within macromolecular machines. *Cell* **104**, 177–190.

## Biography

Smita Patel is a Professor in the Department of Biochemistry at the University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School in Piscataway, New Jersey. Research in her laboratory is focused on understanding the mechanisms of enzymes involved in DNA replication and transcription. She holds a Ph.D. from Tufts University and received postdoctoral training at Pennsylvania State University. Her laboratory is continuing to elucidate the mechanisms of DNA and RNA helicases from phage T7 and hepatitis C virus. In addition, she is interested in understanding the mechanism of transcription and its regulation and has elucidated the kinetic pathway of transcription initiation of a DNA-dependent RNA polymerase and the mechanism of open complex formation using transient state kinetic approaches.

# DNA Ligases: Mechanism and Functions

**Alan E. Tomkinson and John B. Leppard**
*University of Texas Health Science Center at San Antonio, Institute of Biotechnology, San Antonio, Texas, USA*

**DNA ligases are involved in DNA replication, genetic recombination, and DNA repair. These enzymes belong to a larger superfamily of nucleotidyl transferases that also includes RNA ligases and mRNA capping enzymes. Specifically, DNA ligases catalyze phosphodiester bond formation at breaks in the phosphate backbone of duplex DNA. The DNA ligase family can be subdivided into two groups based on cofactor specificity. Prokaryotic enzymes use either nicotinamide adenine dinucleotide (NAD) or adenosine triphosphate (ATP) as a cofactor, whereas viral, archael, and eukaryotic DNA ligases use ATP almost exclusively. DNA ligases share a common core catalytic domain, but the regions flanking the core domain are widely divergent. These unique regions mediate the specific participation of DNA ligases in different DNA transactions.**

## Reaction Mechanism

DNA ligase activity was first identified in 1967 in five different laboratories. In the years that followed, the Lehman laboratory was primarily responsible for elucidating the three-step reaction catalyzed by the NAD-dependent *Escherichia coli* DNA ligase and the ATP-dependent DNA ligase encoded by bacteriophage T4 that is described in this article.

### Adenylation

In the first step, DNA ligase reacts with either nicotinamide adenine dinucleotide (NAD) or ATP to form a covalent enzyme—adenosine monophosphate (-AMP) intermediate (Figure 1). The AMP group is linked via a phosphoramidate bond to a conserved lysine residue that defines the active site of the core catalytic domain (Figure 2A). Formation of the enzyme–AMP intermediate induces a conformational change that is required for the recognition of a nicked DNA substrate in the next step.

### AMP–DNA Intermediate

The second step in the reaction involves the transfer of the AMP group to the 5′-phosphate terminus at the nick in duplex DNA (Figure 1). The previous step leaves the enzyme in an open conformation that exposes the DNA binding site. Specific amino acid residues in the enzyme coordinate the AMP group such that an oxygen atom of the 5′-phosphate in the DNA substrate can attack the phosphoryl group of AMP, generating a DNA–AMP intermediate.

### Phosphodiester Bond Formation

The third and final step of ligation is catalyzed by the nonadenylated form of DNA ligase. In this reaction, esterification of the 5′-phosphoryl group to the 3′-hydroxyl group completes phosphodiester bond formation with the concomitant release of AMP (Figure 1).

## Structure

Gene cloning and, more recently, genome sequencing has led to a rapid growth in the number of DNA ligase genes identified. Alignment of DNA ligase amino acid sequences indicates that these enzymes share a conserved catalytic domain (Figure 2A). Furthermore, a comparison with the catalytic domains of mRNA capping enzymes revealed the presence of six conserved motifs (I, III, IIIa, IV, V, VI) that are characteristic of nucleotidyl transferases. In recent years, our understanding of how the DNA ligase catalytic domain catalyzes DNA joining has been advanced by a combination of approaches that include the use of site-directed mutagenesis to elucidate the role of individual amino acids within the catalytic domain and the determination of the three-dimensional structure of several DNA ligases by X-ray crystallography.

 © 2004, Elsevier Inc. All Rights Reserved. 

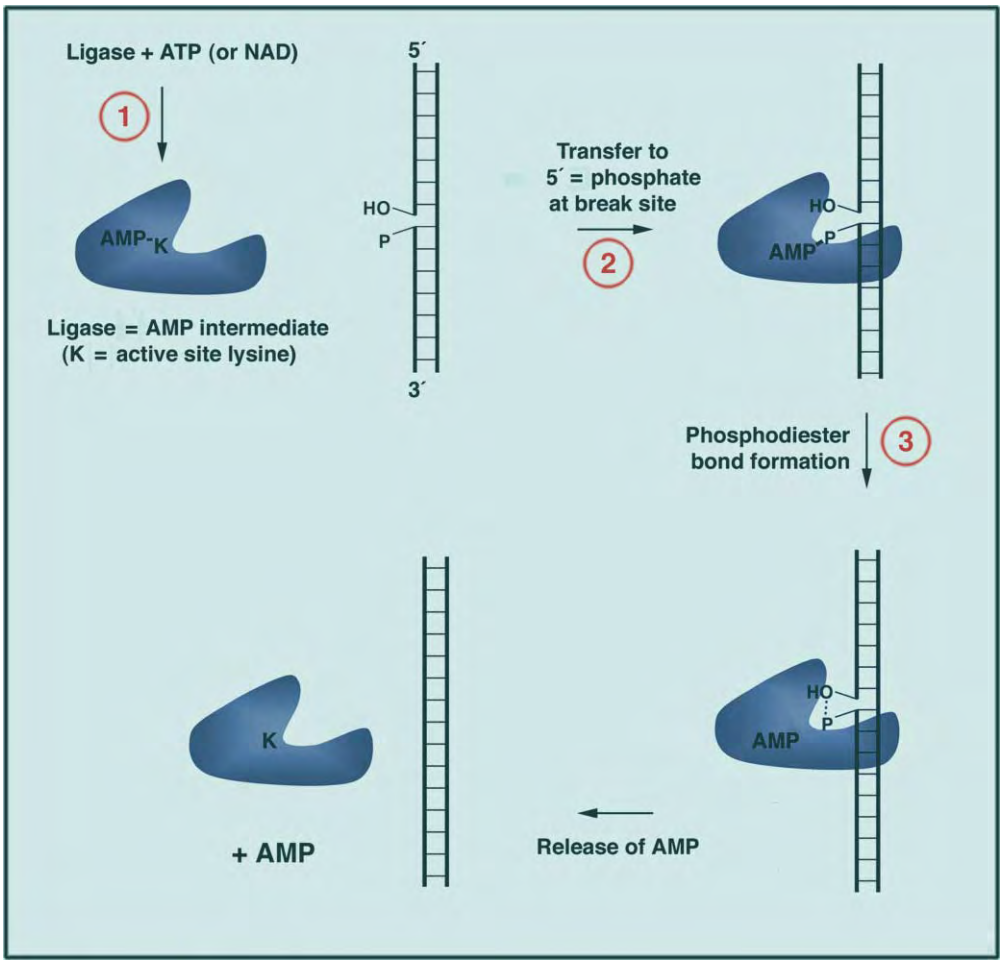



**FIGURE 1** Mechanism of phosphodiester bond formation by DNA ligase. Step 1, DNA ligase interacts with ATP or NAD, forming a covalent enzyme–AMP intermediate. Step 2, after nick recognition, the AMP group is transferred to the 5′-phosphate terminus, forming a high-energy phosphate bond. Step 3, DNA ligase catalyzes phosphodiester bond formation, releasing AMP.

## Crystal Structures

X-ray crystallographic studies of both NAD- and ATP-dependent DNA ligases have provided important insights into the three-dimensional structure of these enzymes. In 1996, the Wigley laboratory solved the first crystal structure of an ATP-dependent DNA ligase, the bacteriophage T7 DNA ligase, in a complex with ATP. More recently, the structure of the enzyme–AMP intermediate formed by the *Chlorella* virus DNA ligase was determined by the Shuman laboratory. A comparison of these structures revealed a conformational change in the DNA ligase catalytic domain that allows binding to nicked DNA. The structures of the ATP-dependent DNA ligases together with those of NAD-dependent enzymes such as *Thermus filiformis* DNA ligase (Figure 2B) and other members of the nucleotidyl transferase superfamily represent snapshots of this family of enzymes at different stages of the catalytic cycle. Thus, they provide a framework for understanding the dynamic conformational changes that occur when nucleotidyl transferases interact with their nucleotide cofactor and polynucleotide substrate.

## Domains Constituting the Core Catalytic Domain

There are two subdomains known as the adenylation domain and the oligomer-binding (OB) fold (Figure 2A) within the DNA ligase catalytic domain. The larger adenylation domain is the minimum region required for formation of an enzyme–AMP intermediate, whereas the OB fold allows the enzyme to bind to DNA and coordinates the ligation event. Residues from motifs I through V line a cleft formed between the two subdomains to generate a positively charged nucleotide-binding pocket. The active-site lysine residue, which is contained within motif I, sits at the bottom of the cleft close to where the adenylation domain and the OB fold are linked. In the nonadenylated form, the enzyme is in an open conformation with the DNA-binding surface of

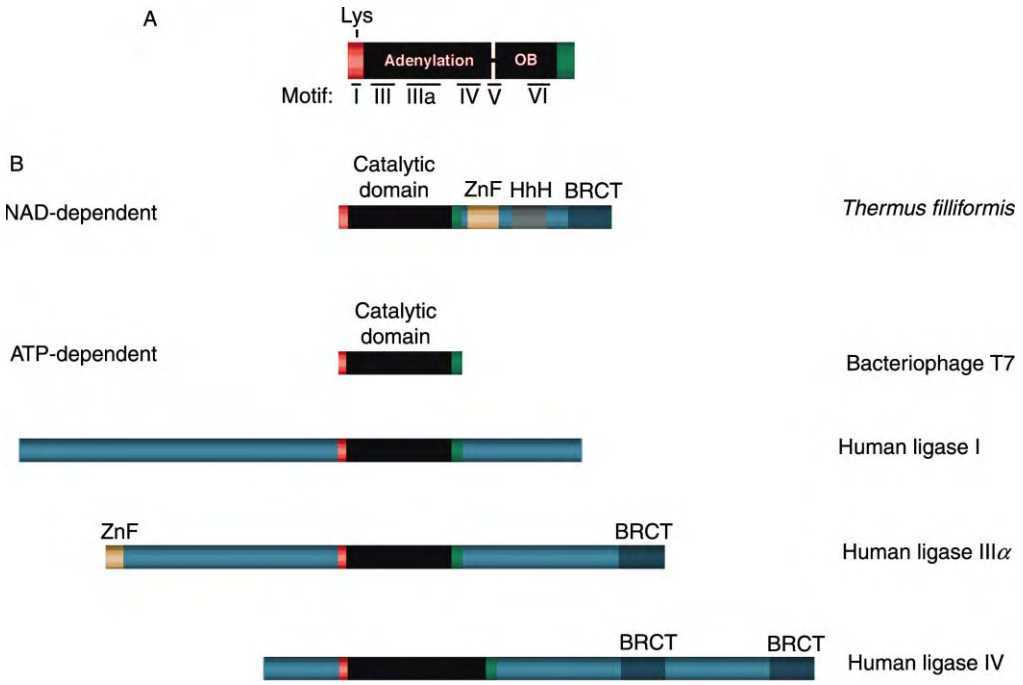



**FIGURE 2** (A) Schematic representation of the core catalytic domain shared by all DNA ligases. (B) Comparison of a prokaryotic NAD-dependent DNA ligase (*Thermus filiformis*) and ATP-dependent DNA ligases from virus (bacteriophage T7) and humans (DNA ligases I, IIIα, and IV). BRCT, BRCA1 C-terminus domain; HhH, helix–hairpin–helix domain; Znf, zinc-finger motif.

the OB fold rotated away from the active site, thus preventing nonproductive DNA binding. Upon adenylation, the enzyme undergoes a conformational change such that the DNA binding surface of the OB fold faces in toward the cleft, making the active site accessible.

### Other Common Domains of NAD-Dependent DNA Ligases

NAD-dependent DNA ligases are relatively uniform in size (~70 kDa). In addition to the adenylation domain and OB fold, they contain several other common motifs (Figure 2B) that are thought to mediate protein–DNA and protein–protein interactions. The zinc-finger (ZnF) and helix–hairpin–helix (HhH) motifs confer DNA binding activity. The BRCT domain, which was first identified in the breast cancer susceptibility gene 1, is probably involved in protein–protein interactions that recruit the enzyme to its site of action.

### Sequences Flanking the Core Catalytic Domain of ATP-Dependent DNA Ligases

Unlike NAD-dependent ligases, ATP-dependent DNA ligases are heterogeneous in size (40–125 kDa) (Figure 2B). The amino acid residues that flank the catalytic domain of ATP-dependent DNA ligases contain a wide variety of sequences that mediate specific protein–protein interactions and protein–DNA interactions (Figure 2B). Intriguingly, recent sequencing studies have identified open reading frames in bacterial genomes whose sequences suggest that ATP-dependent DNA ligase activity may reside in the same polypeptide as nuclease and primase activities.

## Biological Functions

The notion that cells may contain more than one species of DNA ligase was based on initial studies in the Lindahl laboratory describing the properties of various species of DNA ligase in mammalian cell extracts. These biochemical studies led to the cloning of three mammalian genes, *LIG1*, *LIG3*, and *LIG4*, that encode DNA ligases (Table I). More recent genome sequencing has led to the identification of additional DNA ligase genes in organisms such as *S. cerevisiae* and *E. coli* that were thought to have only a single DNA ligase gene. Because DNA joining is required to complete DNA replication, DNA repair, and genetic recombination, it appears likely that the multiple species of DNA ligase have evolved to participate in specific DNA transactions. Insights into the biological functions of the various DNA ligases have been obtained by examining the phenotype of DNA ligase-deficient cells and by identifying protein partners of the DNA ligases (Table I).

**TABLE I**

**Mammalian DNA Ligases**[a]

| Gene | Gene product | Interacting protein | Function |
|---|---|---|---|
| *LIG1* | DNA ligase I | PCNA, Pol$\beta$ | DNA replication, BER, NER, recombination |
| *LIG3* | DNA ligase III$\alpha$ (nuclear) | XRCC1 | Single-strand break repair, BER |
| | DNA ligase III$\alpha$ (mitochondrial) | ? | Single-strand break repair, BER? |
| | DNA ligase III$\beta$ | ? | Postmeiotic repair?, meiotic recombination? |
| *LIG4* | DNA ligase IV | XRCC4 | NHEJ, V(D)J recombination |

[a] BER, base excision repair; NER, nucleotide excision repair; NHEJ, nonhomologous end-joining; PCNA, proliferating cell nuclear antigen; XRCC1, XRCC4, X-ray cross complementing factor 1 and factor 4.

## DNA Replication

The ability to make a copy of their genetic information is essential for all organisms. During DNA replication, DNA joining events are required to link together DNA intermediates known as Okazaki fragments that are generated by discontinuous DNA synthesis on the lagging strand at the replication fork. As expected, inactivation of genes encoding replication proteins, including the replicative DNA ligase, results in cell lethality. Because DNA replication involves the coordinated actions of many different proteins, it seems reasonable to assume that the DNA ligase involved in DNA replication will interact with one or more of the other replication proteins. In mammalian cells, proliferating cell nuclear antigen (PCNA), a homotrimeric ring-shaped clamp protein that was identified as an accessory factor of the replicative DNA polymerase, specifically interacts with the N-terminal region of DNA ligase I. This interaction is critical for the recruitment of DNA ligase I to the sites of DNA replication and for the efficient joining of Okazaki fragments. A similar protein–protein interaction occurs in *E. coli* between the NAD-dependent DNA ligase and $\beta$-clamp, the functional homologue of PCNA, indicating that the interaction between the replicative DNA ligase and the clamp protein is conserved in prokaryotic and eukaryotic DNA replication.

## DNA Excision Repair

Exposure to endogenous DNA-damaging agents such as reactive oxygen species and exogenous DNA-damaging agents such as ultraviolet light results in damage to the nitrogenous bases of DNA. In addition, the DNA replication machinery makes errors that result in mispaired or unpaired nucleotides. Damaged and mispaired nucleotides are removed from the genome by excision repair pathways that share three common steps: (1) excision of the damaged or mispaired DNA, (2) gap-filling DNA synthesis using the undamaged strand as template, and (3) DNA ligation to complete the repair.

The pathways for the repair of damaged bases can be divided into two types based on whether the damage is removed as a nitrogenous base, base excision repair (BER), or as a nucleotide, nucleotide excision repair (NER). In mammalian cells, there are two subpathways of BER, long patch and short patch, that appear to involve two different DNA ligases. Short-patch BER events are mostly completed by DNA ligase III$\alpha$ in a complex with its partner protein X-ray cross complementing factor 1 (XRCC1), whereas long-patch BER is completed by DNA ligase I. NER events are probably completed by DNA ligase I.

In DNA mismatch repair, specific protein factors recognize the mispaired or unpaired nucleotides and direct the excision proteins to the newly synthesized strand. After the removal of a section of newly synthesized DNA containing the mispaired or unpaired nucleotides, the resultant single-strand gap is filled in by a DNA polymerase and repair is completed by a DNA ligase, presumably DNA ligase I.

## Genetic Recombination and Recombinational Repair

Genetic recombination is the major process by which diversity is generated in living organisms. In mammals, exchanges between homologous chromosomes that occur during meiosis contribute to the generation of genetically diverse gametes. It is assumed that DNA ligase I, the replicative DNA ligase, also completes the meiotic recombination events. However, in vertebrates, there is a germ-cell-specific isoform of DNA ligase III, DNA ligase III$\beta$, that may also participate in the completion of meiotic recombination. Alternatively, DNA ligase III$\beta$ may function in DNA transactions in haploid gametes.

Recombination pathways are also critical for the maintenance of genome stability in somatic cells, in particular for the repair of DNA double-strand breaks. This lesion presents a difficult challenge because both strands of the DNA duplex are broken. Recombinational repair pathways can be divided into two types

based on whether they are dependent on DNA sequence homology or not. It is generally assumed that homology-dependent recombinational repair pathways, in particular those involving sister chromatids, are completed by DNA ligase I. In the non-homology-directed repair pathways, the ends of broken DNA molecules are simply brought together by DNA end-bridging factors, processed, and then ligated. Surprisingly, this inaccurate repair pathway, which can result in a wide spectrum of genetic alterations ranging from small deletions to chromosomal translocations, makes a major contribution to the repair of DNA double-strand breaks in mammalian cells. Genetic and biochemical studies have shown that this so-called nonhomologous end-joining (NHEJ) is dependent on DNA ligase IV and its partner protein XRCC4. The same DNA ligase IV/XRCC4 complex is also required for the completion of *** V(D)J recombination, a site-specific recombination mechanism that is required for the rearrangement of immunoglobulin genes to develop a diverse repertoire of antibodies and T-cell receptors.

### Mitochondrial DNA Metabolism

The DNA transactions just described occur in the nucleus. However, the eukaryotic organelles, mitochondria and chloroplasts, contain their own genetic information that must be replicated and repaired. Studies in the Campbell laboratory have shown that nuclear and mitochondrial forms of DNA ligase III are generated by translation initiation at different sites within the same open reading frame encoded by DNA ligase III$\alpha$ mRNA. Although the yeast *S. cerevisiae* lacks a homologue of the *LIG3* gene, it uses the same mechanism to generate mitochondrial and nuclear forms of the Cdc9 DNA ligase.

## Concluding Remarks

Although the basic reaction mechanism catalyzed by DNA ligases was elucidated over 30 years ago, the recent determination of the three-dimensional structure of both NAD- and ATP-dependent DNA ligases has provided exciting new molecular insights into this reaction mechanism. However, structures of DNA ligases interacting with their DNA substrate are needed for a better understanding of the final two steps of the ligation reaction. Because DNA strand breaks are a common intermediate in many different DNA transactions, the study of DNA ligases by genetic and biochemical approaches will continue to provide information about the molecular mechanisms of DNA replication, DNA repair, and genetic recombination. Finally, mutations in DNA ligase genes have been associated with human diseases, highlighting the importance of these enzymes.

### See Also the Following Articles

DNA Base Excision Repair • DNA Ligases: Structures • DNA Mismatch Repair in Bacteria • DNA Mismatch Repair in Mammals • DNA Replication: Eukaryotic Origins and the Origin Recognition Complex • DNA Replication: Initiation in Bacteria • DNA Replication, Mitochondrial • Recombination-Dependent DNA Replication

### Glossary

**adenylation** The reaction in which DNA ligase interacts with ATP or NAD to form a covalent enzyme–adenylate complex.

**motif** An amino acid sequence found to be conserved in various proteins.

**phosphodiester bond** Bond that links deoxynucleotides in DNA, forming the sugar phosphate backbone of the DNA polymer.

**phosphoramidate bond** The covalent bond formed between a phosphoryl group and an amino group. For DNA ligases, the phosphoryl group of AMP is linked to the amino group of the active-site lysine.

### Further Reading

Doherty, A. J., and Suh, S. W. (2000). Structural and mechanistic conservation in DNA ligase. *Mutat. Res.* **28**, 4051–4058.

Lehman, I. R. (1974). DNA ligase: Structure, mechanism and function. *Science* **186**, 790–797.

Lindahl, T., and Barnes, D. E. (1992). Mammalian DNA ligases. *Annu. Rev. Biochem.* **61**, 251–281.

Shuman, S. (1996). Closing the gap on DNA ligase. *Structure* **4**, 653–658.

Timmons, D. J., Singleton, M. R., and Wigley, D. B. *Mutat. Res.* **460**, 301–318.

Tomkinson, A. E., and Mackey, Z. B. (1998). Structure and function of mammalian DNA ligases. *Mutat. Res.* **407**, 1–9.

### Biography

Alan Tomkinson is a Professor in the Department of Molecular Medicine at The University of Texas Health Science Center at San Antonio. His research focuses on the function of eukaryotic DNA ligases in DNA replication, DNA repair, and genetic recombination. He holds a Ph.D. from the University of Newcastle upon Tyne, UK, and received postdoctoral training at the University of California, Berkeley, and the Imperial Cancer Research Fund, UK.

# DNA Ligases: Structures

**C. Kiong Ho**
*State University of New York, Buffalo, New York, USA*

**Mark Odell**
*University of Westminster, London, UK*

**Dimitar B. Nikolov**
*Memorial Sloan-Kettering Cancer Center, New York, USA*

**DNA ligases are enzymes that catalyze the formation of phosphodiester bonds at the site of DNA nicks or breaks. They are responsible for maintaining the continuity of DNA, which is of paramount importance for the survival of all organisms. While many different safeguards exist to repair DNA damage and to accurately replicate or recombine DNA, they all share a common, final procedure, the ligation of DNA. The fundamental nature of this process explains why the DNA ligase family of enzymes is represented in all cellular organisms and in a number of DNA virus genomes. High-resolution crystal structures of DNA ligases and important reaction intermediates illuminate the mechanism of covalent catalysis and provide a framework for understanding the results of preceding investigations of ligase biochemistry.**

## Introduction

The copying and maintenance of the genetic material takes place through the processes of DNA replication, repair, and recombination. In all cases, the final step in these fundamental cellular processes is the sealing of the DNA strands to maintain their fidelity. DNA ligases catalyze the formation of phosphodiester bonds at the site of DNA nicks or breaks via three sequential nucleotidyl transfer steps. In the first step, the enzyme reacts with a high-energy cofactor (ATP or $NAD^+$) to form a covalent ligase-AMP intermediate with a release of pyrophosphate or nicotinamide mononucleotide. In the second step, the ligase-AMP binds a DNA nick and the AMP moiety is transferred from the enzyme to the $5'$ phosphate terminated DNA molecule. The resulting stable DNA adenylate has an inverted $5'–5'$ pyrophosphate bridge structure, AppDNA. In the final step, the ligase catalyzes the nucleophillic attack by the $3'$ hydroxyl group present on the juxtaposed nucleotide of the discontinuous strand, resulting in closure of the gap between the two polynucleotides and release of AMP.

The ATP-dependent ligases and the GTP-dependent capping enzymes are defining members of the superfamily of the covalent nucleotidyltransferases. The first two steps of DNA ligation are analogous to the steps of GTP-dependent mRNA capping that result in guanylate RNA (GpppRNA). The lysine residue located within a conserved Lys-X-Asp-Gly-X-Arg element (named motif I) is the site of covalent linkage of AMP to the ligase and of GMP to the capping enzyme. In addition to this sequence, the members of this enzyme superfamily share five other conserved regions, referred to as motifs III, IIIa, IV, V, and VI. The numbering corresponds to the position within the primary sequence, with motif I being the most proximal to the protein amino terminus, and motif VI to the carboxy terminus. Mutational analysis of the residues within these motifs has documented their essential role in the catalytic activity of both ligases and capping enzymes.

The multi-step, nucleotidyltransferase reactions are catalyzed by single enzymes, which suggests that these proteins undergo dynamic conformational changes to accommodate the distinct substrates utilized at each step. Crystal structures of a number of ATP- and $NAD^+$-dependent ligases, as well as the related mRNA capping enzymes, at intermediate steps along the reaction coordinate have provided insights into the catalytic mechanism of the enzymes.

## Overall Structure of DNA Ligases

DNA ligases are grouped into two families according to their high-energy cofactor requirements for either ATP or NAD. Those utilizing ATP are more widely distributed and are present in eubacteria, bacteriophages, archaebacteria, eukaryotes, and eukaryotic viruses. They vary in size between the 268 amino acid enzyme of *Haemophilus influenzae* and the larger cellular ligases of vertebrates, which are 912 and 844 amino

 © 2004, Elsevier Inc. All Rights Reserved. 

acids, respectively, for the human DNA ligase I and IV enzymes. The first reported three-dimensional structure of an ATP-dependent DNA ligase was that of the bacteriophage T7 enzyme, followed by the structure of the eukaryotic DNA ligase from the *Paramecium Bursari Chlorella Virus*, the smallest known (298 residues) eukaryotic ATP-dependent ligase. In addition, the structures of the GTP-dependent mRNA capping enzymes from *Chlorella* virus and *Candida albicans* were also determined. These studies reveal a common molecular architecture of the ligases and capping enzymes, with a large amino-terminal domain (domain 1) and a smaller carboxy-terminal domain (domain 2) separated by a deep cleft. The amino-terminal domain 1 of the *Chlorella* virus ligase consists of two twisted antiparallel $\beta$-sheets packing against six $\alpha$-helices. This domain contains the nucleotide-binding site, which is generated by the spatial approximation of five of the six conserved nucleotidytransferase motifs (I, III, IIIa, IV, and V), and is located between the two $\beta$-sheets. The carboxy-terminal domain 2 contains an antiparallel $\beta$-sheet and a $\alpha$-helix and folds into an OB-fold, commonly found in nucleic acid-binding proteins. The sixth nucleotidyltransferase motif (motif VI), which is required for covalent enzyme-nucleotide monophosphate (enzyme-NMP) formation, is located within domain 2 in both the ATP-dependent ligases and the mRNA capping enzymes. The conserved spatial arrangement of critical residues and functional groups in the two enzyme classes suggests a common ancestral protein from which the two enzymatic lineages have descended.

Analysis of the structures presented in Figure 2 demonstrates that the *Chlorella* ligase represents the core catalytic element found in all nucleotidyltransferase enzymes. The sequence alignment of known DNA ligases reveals that in addition to this more conserved core element, most members of this enzyme family contain a variety of amino- and carboxy-terminal sequence motifs and domains, which are likely to mediate interactions with other proteins that participate in DNA replication and repair (illustrated in Figure 1). Mammals encode for four distinct DNA ligases (I, III$\alpha$, III$\beta$, and IV). Ligase I contains a proliferating cell nuclear antigen (PCNA) binding motif, which likely functions to coordinate protein-protein interactions at the DNA replication fork. The DNA ligase III gene encodes for two isoforms, III$\alpha$ and III$\beta$, which are produced by alternative splicing and are found in the nucleus and the mitochondria, respectively. Both ligase III isoforms harbor a putative zinc-finger motif, which could facilitate the DNA-enzyme interactions. In addition, the larger ligase III$\alpha$ isoform contains a carboxy-terminal BRCT domain that interacts with XRCC1, a multi-domain protein implicated in base excision-repair. Ligase IV possesses two BRCT domains, and the region between them was shown to bind the repair factor XRCC4 that functions in V(D)J recombination and nonhomologous end joining.

The NAD-dependent DNA ligases are found exclusively in eubacteria, and have a relatively conserved length and sequence. The crystal structures of the NAD-dependent ligase from the thermophilic bacterium *Thermus filiformi* and of the adenylation domain of the *Bacillus stearothermophilus* ligase have been determined. The *T. filiformi* structure documents that the protein contains four discrete domains. Domains 1 and 2 are analogous both in structure and function to

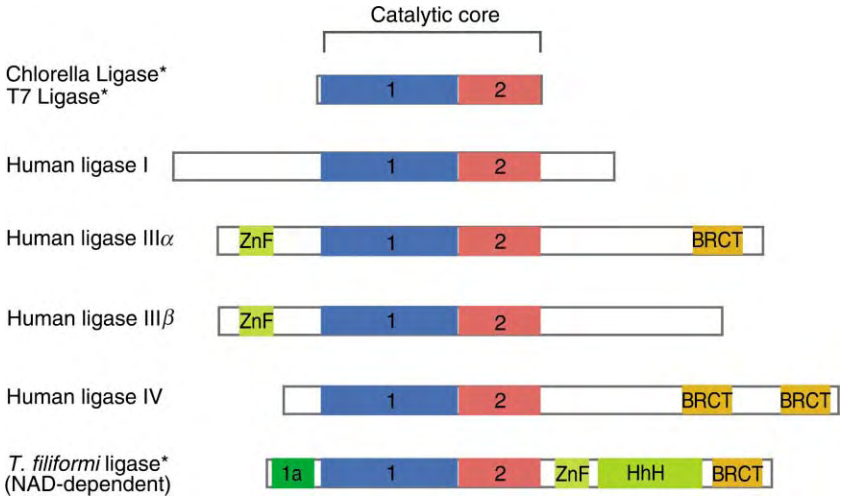



**FIGURE 1** Schematic representation of the domain architecture of known DNA ligases. The domains are color-coded. The asterisk indicates that the high-resolution structure is known. The catalytic core consists of two subdomains: 1 (adenylation), blue; and 2 (oligonucleotide-binding or OB), red. In addition to the catalytic core, some DNA ligases contain other domains and/or motifs, such as zinc fingers (ZnF), helix–hairpin–helix (HhH), and BRCT. Many NAD-dependent DNA ligases also have a small amino-terminal helical subdomain (1a).

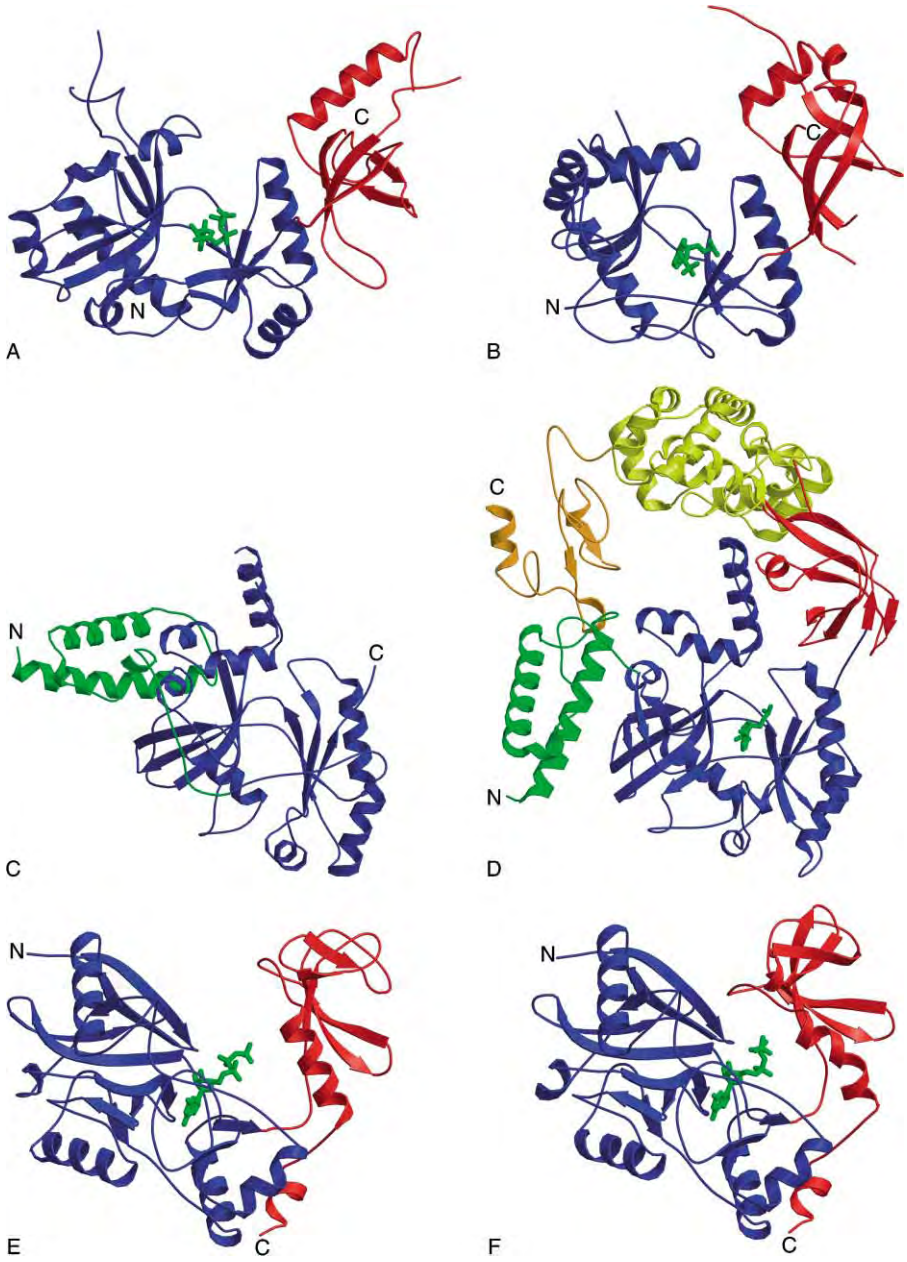



**FIGURE 2** Structures of DNA ligases and the related capping enzymes. The structural domains are color-coded as in Figure 1. The protein amino and carboxy termini are indicated. Bound nucleotides in the active sites are in green. (A) The ATP-dependent DNA ligase of bacteriophage T7, PDB-ID: 1AOI. (B) The ATP-dependent *Chlorella* virus DNA ligase, PDB-ID: 1FVI. (C) The adenylation domain (domain 1) of NAD-dependent DNA ligase from *B. stearothermophilus*, PDB-ID: 1BO4. (D) The NAD-dependent DNA ligase from *T. filiformis*, PDB-ID: 1DGS, 1DGT. (E) *Chlorella* virus capping enzyme open conformation. (F) *Chlorella* virus capping enzyme closed conformation, PDB-ID: 1CKM, 1CKN.

the two catalytic domains of the ATP-dependent enzymes (Figures 1 and 2). Within this region, the five amino-terminal nucleotidyltransferase motifs adopt nearly identical spatial organization to their ATP-dependent counterparts. Domain 1 of the NAD-dependent DNA ligases contains an additional amino-terminal $\alpha$-helical subdomain 1a, which may participate in the recognition and binding of the second nicotinamide mononucleotide moiety of $NAD^+$. Interestingly, domain 2 of the NAD-dependent DNA ligases lacks the motif VI sequence, but still adopts an OB-fold similar to that of the T7 and *Chlorella* ligases. Domain 3 is unique for the NAD-dependent DNA ligase subfamily, and is predicted to be involved in nonsequence-specific DNA binding. It contains a four-cysteine zinc finger and four helix-hairpin-helix (HhH) motifs, which fold around each other to form a compact three-dimensional structure. The carboxy-terminal domain 4 has a BRCT motif similar to those present in mammalian ligases III and IV.

## The Nucleotide-Binding Pocket

The structure of the T7 DNA ligase with bound ATP (Figure 2A) reflects the state of the enzyme prior to the first chemical step of nucleotidyl transfer. The ATP is located in a hydrophobic pocket of the active site with the adenosine base adopting a *syn* conformation relative to the sugar (Figure 3A). The active site lysine (Lys-34) in motif I is positioned close to the $\alpha$-phosphate of the ATP molecule, marking the future site of the covalent AMP-enzyme adduct. Arg-39 in motif I is hydrogen-bonded to the 3′ oxygen of the ribose sugar. Motif III contains two acidic residues, Asp-91 and Glu-93. Asp-91 forms a salt bridge with Arg-39 in motif I, while Glu-93 is hydrogen-bonded to the ribose oxygen at the 2′ position. Two positively charged residues in motif V, Lys-232 and Lys-238, are positioned close to the $\gamma$- and $\beta$-phosphate groups and stabilize the negative charges on the ATP molecule. The aromatic residue Try-149 in motif IIIa stacks against the purine ring of ATP, positioning the adenine base in an optimal orientation for catalysis. The key residues involved in nucleotide binding are conserved both in the ATP- and in the NAD-dependent DNA ligases (as well as in the capping enzymes) and perform similar catalytic functions as visualized in the corresponding *Chlorella* ligase-AMP and *T. filiformi* ligase-AMP structures, suggesting that the reaction mechanism is conserved throughout evolution.

The *Chlorella* ligase–AMP structure reveals that the adenosine-binding pocket is remodeled upon covalent ligase-adenylate complex formation. In contrast to the *syn* conformation of the ATP adenosine bound in the active site of the T7 ligase, the adenosine nucleoside present in the *Chlorella* ligase–AMP complex is in the *anti* conformation (Figure 4A). As a consequence, the contacts between the enzyme and the ribose oxygen are altered during enzyme-adenylate complex formation. The arginine in motif I forms a hydrogen bond to the ribose 2′ oxygen, rather than the 3′ oxygen, while the glutamate side chain in motif III no longer contacts the ribose sugar, leaving the 3′ oxygen without any direct contacts with the protein. The observation that both the *C. albicans* capping enzyme-GMP and NAD-dependent *T filiformi* ligase-AMP (Figure 3B) intermediates adopt an *anti* conformation suggests that the change in conformation from *syn* to *anti* during enzyme-NMP formation is a general feature of the nucleotidyltransferase superfamily. The wider implication of this structural rearrangement is that the ribose 3′ oxygen is free to directly coordinate the 5′ nucleotide phosphate group of the nucleic acid substrate.

## Mechanism of Nucleotidyl Transfer

The first direct evidence for conformational change during the covalent enzyme–NMP complex formation was evinced by crystal structures of two distinct conformations of the *Chlorella* virus mRNA capping enzyme bound to GTP. The two states are referred to as "open" and "closed" capping enzyme-GTP complexes (Figures 2E and 2F). The open conformation corresponds to the enzyme making an initial or transient contact with GTP. The closed complex structure represents the conformation in which the enzyme is stably bound to GTP, and is committed to the nucleotidyl transfer reaction. The two structural forms differ in the relative position of domain 2 with respect to the GTP-binding pocket: in the open complex, the cleft between the domains is wider and as a result the residues within motif VI are unable to make direct contact with GTP. In the closed complex structure, the positively charged amino acids in motif VI make direct contact with the $\beta$- and $\gamma$-phosphates of GTP, suggesting that these conserved residues function to facilitate the release of pyrophosphate after the formation of the lysyl–NMP

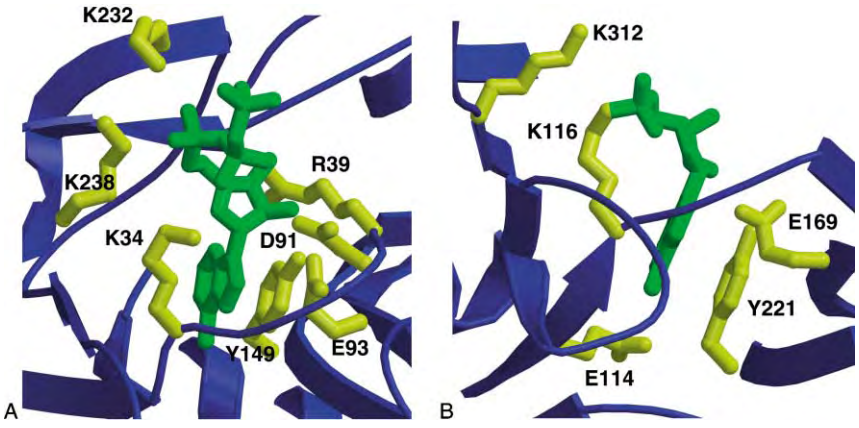



**FIGURE 3** The nucleotide-binding pocket of DNA ligases. The ligase adenylation domain (domain 1) is in blue; the side chains of important residues (discussed in the text) are in yellow; and the bound nucleotide is in green. (A) ATP bound in the active site of the bacteriophage T7 DNA ligase. (B) AMP covalently bound to the active-site lysine of the NAD-dependent *T. filiformis* DNA ligase.

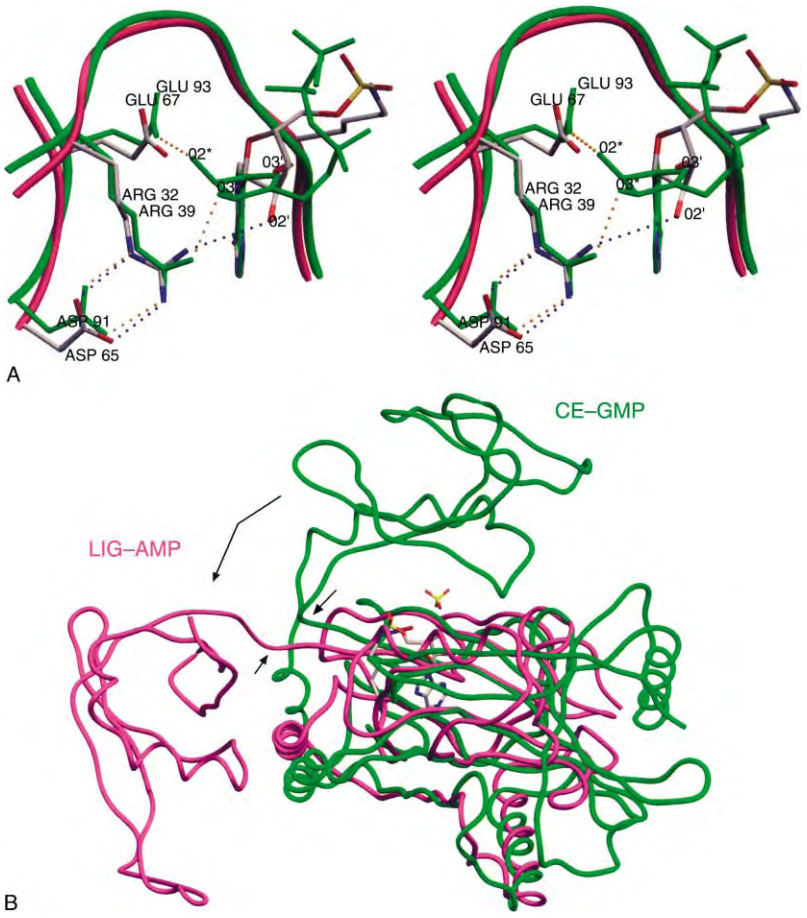



**FIGURE 4** Conformational changes in DNA ligases during catalysis. (A) Remodeling of the adenosine-binding pocket after ligase–adenylate formation. Stereoview of the nucleotide-binding pocket of *Chlorella* ligase (purple, with side chains in CPK) highlighting interactions of motif I and III side chains with the ribose sugar of the lysyl-adenylate adduct. The superimposed equivalent structural elements of the T7 ligase–ATP complex are in green. Hydrogen bonds are denoted by dashed lines. (B) Comparison of the *Chlorella* virus ligase (purple) and capping enzyme (green) structures. The structures are superimposed with reference to the nucleotide-binding pocket of domain 1. The lysyl–AMP adduct and the sulfate in the ligase are shown. The guanylate and a sulfate are similarly positioned in the capping enzyme structure. The figure highlights the large movement of domain 2 from the closed state (capping enzyme) to the wide-open conformation (ligase) that exposes a DNA-binding surface. The principal flexion points within the inter-domain linkers (in motif V) are indicated by the short arrows. Reproduced with permission from Odell, M., Sriskanda, V., Shuman, S., and Nikolov, D. B. (2000). Crystal structure of eukaryotic DNA ligase–adenylate illuminates the mechanism of nick sensing and strand joining. *Mol. Cell.* **6**, 1183–1193 with permission from Elsevier.

covalent complex. The requirement of the equivalent motif VI residues in the ATP-dependent ligases for enzyme activity strongly suggests that they undergo a similar conformational change during the first step of the reaction.

While the structure of a ligase bound to DNA has yet to be determined, the ligase-AMP structures of the *Chlorella* virus DNA ligase provide valuable insight into the mechanism of nicked duplex DNA recognition. Occupation of the adenylate binding site is known to be crucial to nick recognition, and in this "DNA-binding-competent" state, domain 2 of the *Chlorella* ligase-AMP complex is pivoted away from domain 1 via a bending in the flexible inter-domain linker region (Figure 4B). The cleft between domains 1 and 2 is substantially wider than in the open complex form of the capping enzyme-GTP complex, and this ligase-adenylate structure is therefore referred to as the "wide-open" complex. The "wide-open" conformation is proposed to expose the DNA-binding surface on domain 1 allowing the 5′ phosphate of the nicked DNA substrate to make direct contact with the adenylation site on the enzyme. Analysis of the surface properties of the *Chlorella* ligase further suggests that the ligase–AMP is ready to bind DNA. In this conformation the electrostatically positive surface of domain 1 favors interaction with the negatively charged phosphodiester backbone of DNA. The relatively flat surface of the ligase molecule (Figure 4B) could allow direct interactions with one face of the DNA double helix. The remodeling of the active site leaves the 3′ oxygen of the ribose sugar free to participate directly in the coordination of

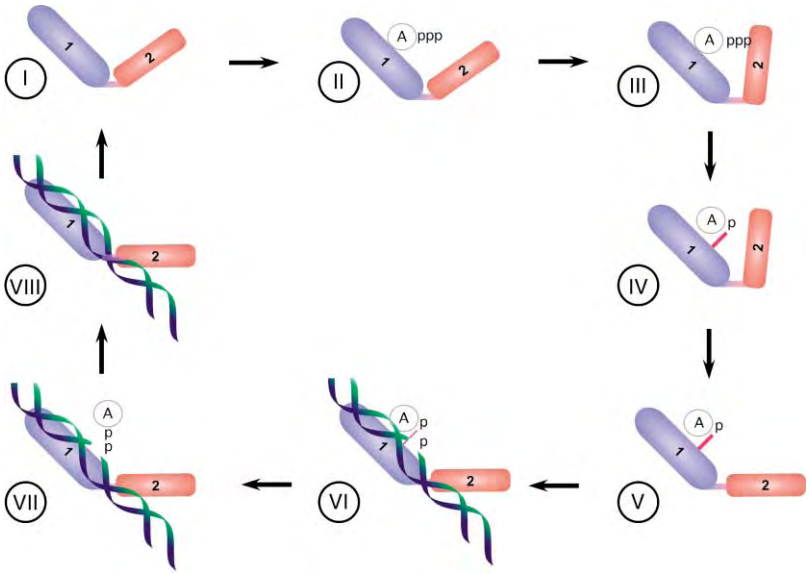



**FIGURE 5** Schematic model of ATP-dependent DNA ligation showing the individual catalytic steps and the corresponding conformational changes in the enzyme. Only the catalytic core (domains 1 and 2) of the DNA ligase is shown. See text for details.

the 5′ phosphate of the DNA substrate. This indicates that only catalytically licensed forms of DNA ligases will be able to stably interact with a nicked DNA substrate. The structural data also suggest that domain 2 might not directly participate in the subsequent catalytic events that result in the adenylation of DNA (step 2) and in phosphodiester bond formation (step 3).

A model of ATP-dependent ligation consistent with the known ligase and capping enzyme structures is shown in Figure 5. Specifically, the structural data support a common mechanism of catalysis by nucleotidyltransferases that is carried out through a series of conformational changes induced by substrate binding. In the first step, the energy cofactor, ATP, binds to the open form of enzyme (state I on Figure 5). The binding of this substrate and/or of a divalent cation promotes domain closure, bringing the positively charged residues of the OB-fold into direct contact with the pyrophosphate leaving group (II and III). As a result of the structural rearrangement, the active site lysine is positioned to make an in-line attack on the $\alpha$-phosphate of the ATP (III). The following formation of the ligase-AMP complex is accompanied by release of pyrophosphate (IV). The ligase undergoes a transition from a "closed" (IV) to a "wide-open" complex (V). This transition is an example of ping-pong kinetics where the active site is initially modified by the first substrate (ATP) to permit recognition of the second substrate (DNA). While structures of DNA ligases in complex with DNA are not yet reported, it is hypothesized that the wide-open complex would allow the nicked duplex DNA to be recognized and bound by the positively charged surface on the nucleotidyltransferse domain 1 (VI) and stabilized by bonding of the 5′ phosphate of DNA by the 3′ oxygen of the NMP sugar moiety, facilitating the transfer of AMP to the 5′-phosphate terminated DNA strand (VII). Once the DNA-adenylate is formed, the enzyme remains bound to the activated nick and catalyzes its sealing via an attack of the 3′ OH on the 5′ adenylated DNA, producing a phosphodiester linkage with the concomitant release of AMP (VIII). It should be emphasized that this model is consistent with ligation of a single nick in an otherwise duplex DNA molecule. The ligation of double-stranded DNA breaks is catalyzed by the mammalian DNA ligase IV, and requires additional proteins to stabilize the free DNA ends. The functional catalytic complex in this case may contain two molecules of DNA ligase, each sealing a single DNA strand.

## See Also the Following Articles

ATP Synthesis in Plant Mitochondria: Substrates, Inhibitors, Uncouplers • DNA Ligases: Mechanism and Functions

## Glossary

**ATP** Adenosine triphosphate. An adenosine-containing nucleotide that harbors high-energy phosphate bonds and is used to transport energy to cells for biochemical processes through its hydrolysis.

**covalent nucleotidyltransferase** Enzyme that reacts with nucleotides and transfers the nucleoside moiety to an acceptor molecule

through the formation of a covalent nucleoside monophosphate (NMP)-enzyme intermediate.

**mRNA cap** Structure found at 5′ end of the eukaryotic mRNAs, consists of 7-methylguanosine linked to the end of the transcript via a 5′–5′ triphosphate bridge (m7GpppN). The cap structure enhances mRNA stability, splicing of pre-mRNAs, and initiation of protein synthesis.

**NAD** Nicotinamide adenine dinucleotide (oxidized form). A coenzyme present in all cells that assists enzymes by accepting electrons during metabolic reactions. Acts as an energy donor in bacteria by virtue of a high-energy P–P bond.

## Further Reading

Cherepanov, A. V., and de Vries, S. (2002). Dynamic mechanism of nick recognition by DNA ligase. *Eur. J. Biochem.* **269**, 5993–5999.

Doherty, A. J., and Suh, S. W. (2000). Structural and mechanistic conservation in DNA ligases. *Nucleic Acids Res.* **28**, 4051–4058.

Hakansson, K., Doherty, A. J., Shuman, S., and Wigley, D. B. (1997). X-ray crystallography reveals a large conformational change during guanyl transfer by mRNA capping enzymes. *Cell* **89**, 545–553.

Lee, J. Y., Chang, C., Song, H. K., Moon, J., Yang, J. K., Kim, H. K., Kwon, S. T., and Suh, S. W. (2000). Crystal structure of NAD(+)-dependent DNA ligase: Modular architecture and functional implications. *EMBO J.* **19**, 1119–1129.

Odell, M., Sriskanda, V., Shuman, S., and Nikolov, D. B. (2000). Crystal structure of eukaryotic DNA ligase–adenylate illuminates the mechanism of nick sensing and strand joining. *Mol. Cell.* **6**, 1183–1193.

Shuman, S. (1996). Closing the gap on DNA ligase. *Structure* **4**, 653–656.

Subramanya, H. S., Doherty, A. J., Ashford, S. R., and Wigley, D. B. (1996). Crystal structure of an ATP-dependent DNA ligase from bacteriophage T7. *Cell* **85**, 607–615.

Timson, D. J., Singleton, M. R., and Wigley, D. B. (2000). DNA ligases in the repair and replication of DNA. *Mutat. Res.* **460**, 301–318.

Tomkinson, A. E., and Mackey, Z. B. (1998). Structure and function of mammalian DNA ligases. *Mutat. Res.* **407**, 1–9.

## Biography

C. Kiong Ho is an Assistant Professor in the Department of Biological Sciences at the State University of New York, Buffalo. His laboratory is taking a molecular and biochemical approach to study mRNA processing events (mRNA capping and RNA editing) in parasitic protozoa.

Mark Odell studied DNA ligase enzymology for his doctorate at the University of Oxford, then subsequently in the USA at the Sloan-Kettering Institute with Professor Stewart Shuman. He now runs a research group at the University of Westminster, London, using structure and biochemistry to understand how proteins function.

Dimitar B. Nikolov is an Associate Member of the Memorial Sloan-Kettering Cancer Center and an Associate Professor at the Weill Medical College of Cornell University in New York City. He holds a Ph.D. degree from The Rockefeller University. His laboratory uses X-ray crystallography combined with other biophysical methods to study the structure, function, and mechanism of action of proteins.

# DNA Methyltransferases, Bacterial

**Albert Jeltsch**
*International University Bremen, Bremen, Germany*

**Richard I. Gumport**
*University of Illinois, Urbana, Illinois, USA*

**DNA methylation has a number of important roles in bacteria including the control of gene expression, DNA replication, and the cell cycle. In addition, it is involved in mismatch repair and protection of bacteria from foreign DNA in restriction modification systems. DNA methyltransferases (MTases) are the enzymes that methylate DNA. They deposit methyl groups on DNA at the N6-position of adenine, or the N4- or C5-positions of cytosine in a sequence-specific reaction using S-adenosyl-L-methionine (AdoMet) as the methyl group donor. Their reaction mechanism includes rotating the target base completely out of the DNA helix in a biphasic process, where fast flipping of the base out of the double helix is followed by a slower binding of the flipped base into a hydrophobic pocket of the enzyme. DNA MTases comprise two structural domains: the larger domain contains the cofactor-binding site and the binding pocket for the flipped base and the smaller domain is responsible for most of the sequence-specific contacts of the enzyme to the target site. The structures of large domains from all known DNA MTases are similar, whereas the small domains are more heterogeneous in sequence and structure. DNA MTases are an attractive model system to study how proteins recognize specific sequences of DNA and how the specificity of DNA recognition changes during molecular evolution. In addition, they illustrate how the biochemical properties of the enzymes are related to their biological functions. In this article, we shall describe the biological roles of DNA methylation in prokaryotes, discuss the chemistry of the enzymatic methylation reaction performed by the DNA methyltransferases (the focus of the review), and finally discuss aspects of the enzymology of this fascinating family of enzymes that reveal how these molecular machines perform their complicated biochemical tasks.**

## Introduction

After their discovery during the 1940s, methylated nucleobases have been found in the DNA of most species. DNA methyltransferases (MTases) produce three types of methylated bases, C5-methylcytosine, N4-methylcytosine, and N6-methyladenine (Figure 1). Based on the identity of the acceptor atom for the methyl group, C-MTases forming C5-methylcytosine can be distinguished from N-MTases forming N6-methyladenine and N4-methylcytosine. Whereas C5-methylcytosine occurs in higher eukaryotes and prokaryotes, the N-modified bases (N4-methylcytosine and N6-methyladenine) are present only in prokaryotes and some lower eukaryotes. Usually, DNA MTases are DNA sequence specific. In higher eukaryotes, cytosine methylation occurs mainly at CG sequences, and in plants also at CNG sequences. Such DNA methylation leads to chromatin condensation and silencing of gene expression. In prokaryotes, DNA methylation is involved in the control of gene expression, but it has a multitude of additional roles including control of initiation of DNA replication, correction of errors in DNA replication, and modulation of the destruction of DNA by restriction endonucleases.

## Biological Function of DNA Methylation in Prokaryotes

### Molecular Recognition of the Presence of the Methyl Groups on the DNA

DNA methylation adds an extra layer of information, viz., epigenetic information, to the genome that extends the intrinsic genetic information encoded in the unmodified sequence composed of the four usual Watson–Crick base pairs. In this sense, it can be compared to the different written diacritic marks in the French language, which are not required to read a word but rather define how the words are pronounced. Thus, diacritic signs do not carry essential information for the meaning of text, but impart important information beyond that level. Likewise, DNA methylation adds information that influences the stability, regulation, and expression of the encoded genetic information.

 © 2004, Elsevier Inc. All Rights Reserved. 

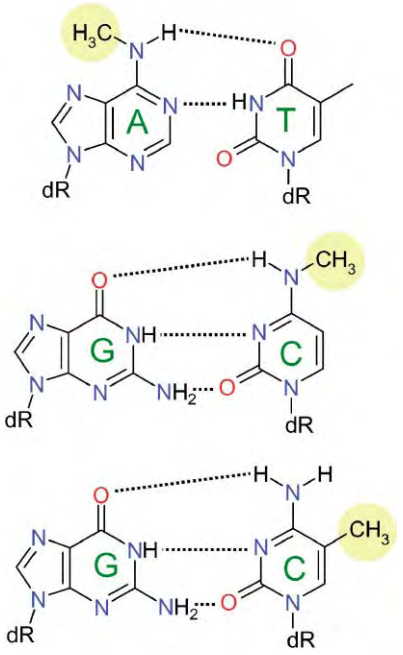



**FIGURE 1** Structures of 6-methyladenine and of 5- and 4-methylcytosine in the context of an AT and GC base pairs. In all cases, the methyl group is located in the major group of the DNA and it does not interfere with the Watson–Crick base pairing.

Methylation of the DNA at the cytosine-C5, N4, and adenine-N6 positions does not interfere with Watson–Crick base pairing (Figure 1). It represents a minimal change of the molecular structure of the DNA. However, the methyl groups are positioned in the major groove of the DNA, a region where many proteins recognize the sequence of the DNA by formation of specific contacts to the edges of the base pairs. Such recognition can be severely disturbed by the presence of a single methyl group in the major groove as illustrated by the complexes of restriction endonucleases with specific DNA. These enzymes form a very tight interface with their target DNA, stabilized, in part, by several base-specific interactions. Methylation of the target DNA base sequence prevents close approximation of the enzyme and the DNA through steric hindrance, thereby effectively preventing DNA cleavage by the restriction enzyme. Thus, the introduction of a single methyl group onto a DNA base can have a profound effect on the biological functions of the modified sequence.

## The Molecular Logic of DNA Methylation

The effect of DNA MTase action is to give DNA a methylation pattern that will affect its biological function. In prokaryotes, we can discern several levels of biological responses to methylation.

### Sequence Context of DNA Methylation

Most prokaryotic DNA MTases are parts of restriction/modification (RM) systems, which are widely distributed in the bacterial and archeal kingdoms. These systems comprise two enzymes, a restriction endonuclease that specifically recognizes and cleaves DNA usually within or near short, often palindromic sequences. The cellular DNA is protected from cleavage by the MTase, because it modifies the DNA within the same sequence and prevents endonuclease action. Since different RM systems have different recognition sequences, the methylation pattern basically imprints a bar code on the DNA that allows the bacterium to distinguish between its own and foreign DNA. Since DNA from bacteriophages is often not modified at the sites of the resident RM systems, these systems efficiently protect against bacteriophage infection. In addition, they serve to control the uptake and incorporation of foreign DNA from any source into the bacterial chromosome. Because RM systems distinguish between self and nonself DNA and protect bacteria from phage infection, they can be compared, in biological function, to the immune system of higher eukaryotes. Like the immune system, RM systems are also involved in a molecular arms race between the invader and the host, and bacteriophages have evolved many antirestriction systems, such as multispecific MTases that modify the phage genome at many sites, and thereby provide a broad protection against the endonucleases of different RM systems.

### Regulation by Hemimethylation

***Coordination of DNA Replication and Cell Division*** In addition to the MTases that are part of RM systems, solitary MTases also occur. These enzymes are not accompanied by a restriction enzyme and are exemplified by the *Escherichia coli* dam or *Caulobacter cresentus* CcrM MTases. Both modify adenine residues; their palindromic recognition sequences are GATC and GANTC, respectively. Therefore, both strands of the DNA usually carry a methyl group, and the duplex sites are fully methylated (5′-G$^{m}$ATC-3′/3′-CT$^{m}$AG-5′ in the case of dam). Since only unmodified nucleotides are incorporated during DNA replication, the newly synthesized strand of the DNA lacks methyl groups. Therefore, replication transforms the DNA methylation status from a fully methylated into a hemimethylated state. In *E. coli*, the dam sites in most parts of the genome are remodified rapidly after replication. In contrast, the SeqA protein tightly binds to the origin of replication and protects it from being remethylated for several minutes. During this time, the origin cannot initiate a new round of DNA replication thereby coupling DNA replication and cell division. Thus, in

this system the hemimethylated status of the dam sites determines whether DNA replication will occur again.

***Postreplicative Mismatch Repair*** As described, DNA replication generates hemimethylated dam sites in *E. coli*. Such strand-specific methylation distinguishes the parental and daughter DNA strands; the residual methylation always marks the parental strands. Strand-specific asymmetric methylation is the basis for a directed repair of replication errors by the *E. coli* Mut HLS system. Mismatched base pairs resulting from erroneous nucleotide incorporation by the DNA polymerase are recognized by the MutS protein or homologues in a mechanism that is conserved from *E. coli* to man. In *E. coli*, MutH, a partner in a complex with MutS and MutL, specifically cleaves the DNA at hemimethylated GATC sites in the unmodified, newly synthesized daughter strand. This allows differentiating the erroneous new strand from the original stand and directs the repair process to the proper strand. Therefore, strand discrimination and postreplicative mismatch repair in *E. coli* and other dam-positive bacteria is connected to dam methylation. Loss of the dam methylation generates a hypermutational phenotype that contributes to pathogenicity of *Neisseria meningitides*. Other mechanisms of strand discrimination, which do not depend on DNA methylation, are operative in higher eukaryotes and bacteria not having dam methylation.

***Cell-Cycle Control*** *Caulobacter cresentus* is an $\alpha$-proteobacterium with a well-controlled cell cycle that is coordinated to a phase transition between mobile swarmer cells and stalk cells. Its genome changes from the fully methylated to the hemimethylated state during DNA replication. The CcrM Mtase in *Caulobacter cresentus* is among the proteins whose expression is regulated during the cell cycle such that it is present only during a short time near the end of S phase. The CcrM MTase is a master regulator gene, because the change between the hemimethylated and fully methylated state itself regulates a variety of other proteins, including the CtrA global regulator.

### Regulation of Gene Expression by Methylation Pattern

Examples of gene regulation by the dam MTase in *E. coli* were detected several years ago, when it was shown that the pap operon in uropathogenic *E. coli* strains is regulated by differential methylation. Analyses of the *E. coli* genome showed that there are stably undermethylated sets of GATC sites whose position can vary with environmental conditions. Most likely, these sites are blocked by other proteins tightly bound to the DNA such that the dam enzyme cannot gain access. Recent whole-genome expression profiles have revealed a much higher number of genes whose expression is regulated by dam methylation. Regulation of gene expression by dam methylation contributes to phase variations of *E. coli* and other $\gamma$-proteobacteria. This is the most likely reason that dam-negative strains of *Salmonella thyphimurium* are not pathogenic and can serve as live vaccines.

## Chemistry of DNA Methylation

DNA MTases use S-adenosyl-L-methionine (AdoMet) as the donor of an activated methyl group. The activated methylsulfonium of AdoMet can be attacked by a nucleophile to effect the methylation (Figure 2). However, methylation of either the exocyclic amino groups of adenine and cytosine or the C5-position of cytosine is a difficult chemical task, because these positions are not intrinsically nucleophilic. For instance, chemical alkylation of adenine and cytosine occurs at positions 3 of cytosine and 1 or 7 of adenine rather than the atoms modified by the MTases. To overcome this intrinsic lack of reactivity, the two families of DNA MTases, N-MTases and C-MTases, have evolved highly sophisticated reaction mechanisms. Both mechanisms have in common the necessity for close contact between the

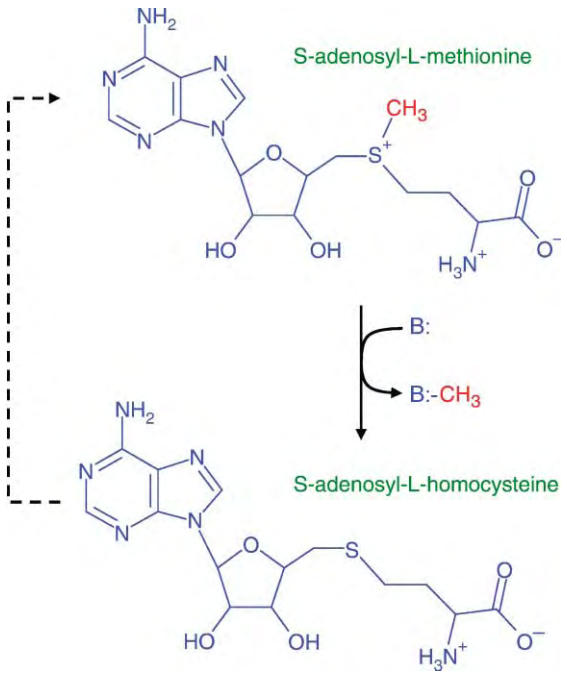



**FIGURE 2** The structure of AdoMet, the cofactor of the methylation reactions catalyzed by DNA MTases. In the methylation reaction AdoMet is converted into S-adenosyl-L-homocysteine (AdoHcy). AdoMet can be regenerated from AdoHcy in a multistep pathway depending on tetrahydrofolate and Coenzyme-B12 that employs serine, which itself can be derived from 3-phosphoglycerate or glycine as the ultimate donor of a C1-group.

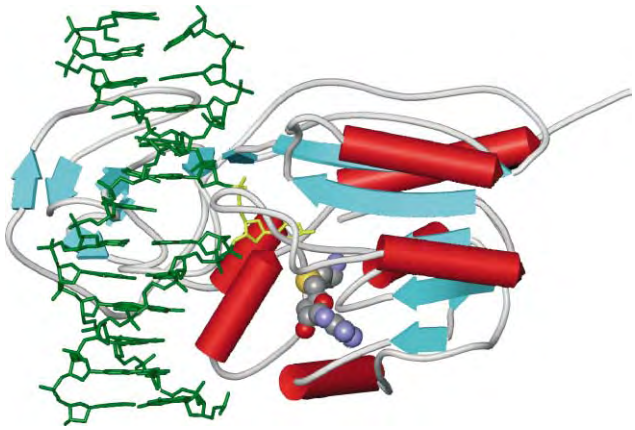


**FIGURE 3** Structure of M.HhaI (gray, blue, and red) showing the target base (yellow) flipped out of the DNA helix (black). The bound cofactor AdoMet (purple) is displayed in its space-filling form.

enzyme active site and the target base. Such proximity is not possible while the base is located within the DNA double helix. In seminal studies, X. Cheng, S. Klimasauskas, R. Roberts, and co-workers demonstrated that the cytosine-C5 Mtase HhaI completely flips its target base out of the DNA double helix during catalysis – an unprecedented, dramatic conformational change in the DNA (Figure 3). Later, similar mechanisms were observed with another cytosine-C5 MTase (HaeIII) and more recently with an adenine-N6 MTase (TaqI).

## Reaction Mechanism of Cytosine-C5 MTases

Methylation of the C5-position of cytosine follows a two-step reaction mechanism (Figure 4) that resembles a Michael addition and is analogous to the mechanism used by thymidylate synthase to convert dUMP to dTMP. In the first step, a cysteine residue in the active site of the DNA MTase performs a nucleophilic attack on the C6-position of the cytosine that is flipped out of the DNA and bound in a hydrophobic pocket of the enzyme. Thereby, a transient carbanion is generated, which is stabilized by a protonated glutamic acid residue that hydrogen-bonds to the flipped base and protonates the N3 atom of the base. Since the carbanion has a high negative charge density at position 5, the activated base can attack the methyl group of the cofactor yielding a stable intermediate comprising a covalently linked complex of the methylated DNA and the MTase. The catalytic cysteine and glutamic acid residues are located in two highly conserved amino acid motifs found in cytosine-C5 MTases, the PCQ motif and the ENV motif also called motif IV and motif VI. The second step of the reaction resolves the covalent intermediate. It is initiated by deprotonation of C5 catalyzed by an unknown proton acceptor; deprotonation leads to the elimination of the cysteine residues and re-establishment of aromaticity.

This mechanism is supported by several lines of evidence. The covalent complex has been detected in crystal structures and in biochemical studies, the catalytic cysteine and glutamic acid residues are highly conserved in all cytosine-C5 MTases, and mutational analyses have supported their importance. Moreover, MTases effect the methylation reaction by positioning of the substrate and the cofactor in the active site and by tight binding to the transition state of the reaction.

## Reaction Mechanism of N-MTases

Chemically, methylation of either adenine and cytosine residues at their exocyclic amino group is similar, because in each case the substrate amino group is part of an electron-poor, heterocyclic, aromatic system. The reaction is difficult, because the free electron pairs formally present at the amino groups are delocalized into the aromatic system and not readily available for nucleophilic attack on the AdoMet. The catalytic center of the MTases that acts on exocyclic amino groups (N-MTase) is formed by a (D/N/S)PP(Y/F) motif. The reaction mechanism of the N-MTases (Figure 5) is illuminated by the structure of the TaqI adenine-N6 MTase. The carboxyamide group of the active site asparagine and the backbone carbonyl group of the second proline residue of the active site tetrapeptide contact the amino group of the flipped adenine target. Since the hydrogen acceptor groups are presented in a tetrahedral geometry,

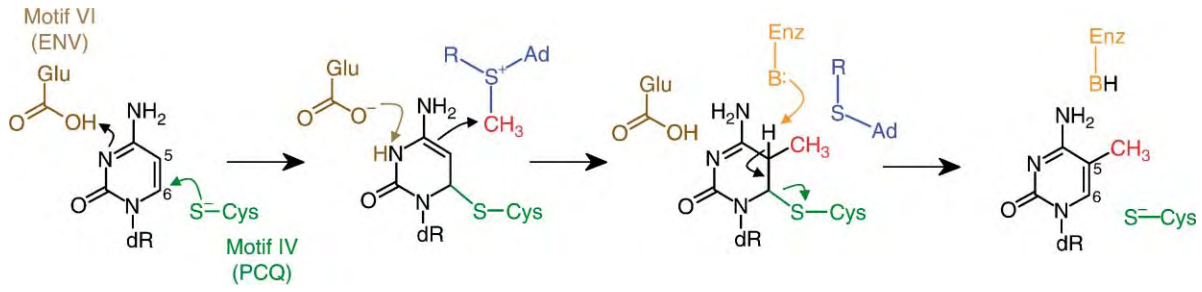



**FIGURE 4** Schematic drawing of the catalytic mechanisms of C-methylation. The conserved motifs ENV and PCQ are indicated.

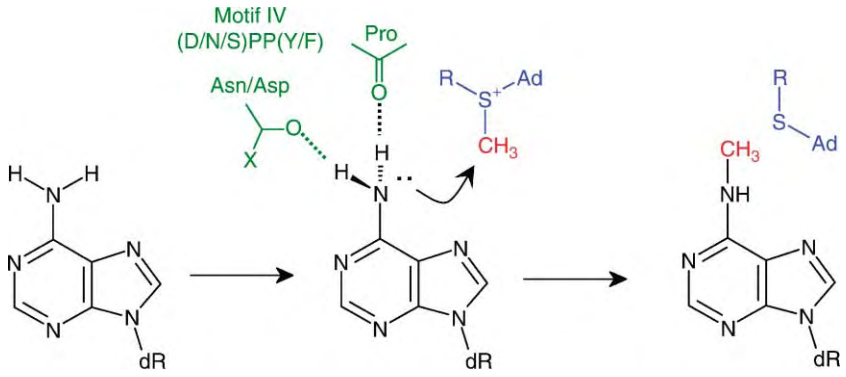



**FIGURE 5** The catalytic mechanism of N-methylation. The conserved catalytic motif (D/N/S)PP(YF) is indicated.

formation of the hydrogen bonds induces a change in hybridization of the amino group from $sp^2$ to $sp^3$. This localizes the free electron pair and makes it available for the nucleophilic attack on the methyl group of the AdoMet. This mechanism is consistent with results from site-directed mutagenesis studies with many different DNA N-MTases, which demonstrate the importance of the active site (D/N/S)PP(Y/F) motifs. In addition, positioning of the base and the cofactor appear to be critical elements for catalysis by N-MTases.

It is interesting that the recognition of the flipped target base by the MTase is not necessarily precise; adenine-N6 MTases modify cytosine residues at position N4 and cytosine-N4 MTases modify adenine residues at position N6. Although these reactions occur with reduced efficiencies, they prove that the catalytic mechanisms of adenine-N6 and cytosine-N4 MTases are similar. Surprisingly, it has been shown recently that HemK enzymes, which are conserved from bacteria to human and strongly resemble adenine-N6 MTases in all their characteristic primary sequence motifs, are Glutamine-N5 methyltransferases. This unexpected finding demonstrates that the (D/N/S)PP(Y/F) tetrapeptide is a versatile active-site motif that supports methylation of various amino groups that are conjugated to an electron poor $\pi$-conjugation system, viz., the purine ring in adenine-N6 methylation, the pyrimidine ring in cytosine-N4 methylation, or a carbonyl group of glutamine-N5 methylation. It will be interesting to see whether (D/ N/S)PP(Y/ F)-containing enzymes have even more substrates, for instance if they can support methylation of arginine.

## Mechanism of Base Flipping

Base flipping is a prerequisite for the catalytic mechanism of all DNA MTases; all structures of DNA MTases in complex with DNA solved so far show base flipping (Figure 3). In addition, numerous biochemical studies with different MTases demonstrate base flipping in solution. Three lines of biochemical evidence support a base flipping mechanism: (1) DNA MTases often bind substrates that contain a base mismatch at the target site more tightly than a normal substrate, because base flipping requires disruption of the base pairing between the target base and its partner base. (2) 2-Aminopurine, a base analogue that is fluorescent in solution but is highly quenched in the DNA by base stacking to its neighbor bases can be incorporated in the DNA as target base. Base flipping destroys the stacking interactions and sometimes leads to a strong and specific increase of fluorescence that is correlated with the flipping process. (3) The target base of DNA MTases shows a high propensity to react with the protein during cross-linking experiments. This result suggests that the base is flipped out of the helix and thereby available for much closer contact with the protein than it could if it remained inside the DNA helix. However, one should bear in mind that none of these assays is sufficient to prove or disprove unequivocally base flipping.

Our understanding of base flipping is limited by the high efficiency of the process, because so far no structural information is available for the specific DNA MTase complex prior to base flipping. The equilibrium favoring the flipped-base state is related to structural relaxations of the protein–DNA complex that prevent back-rotation and reinsertion of the flipped base into the DNA helix. Interestingly, these structural changes are different in all three complexes of DNA MTase with specific DNA available so far. In the case of M.HhaI, the DNA retains an almost perfect B-DNA-like conformation and the enzyme inserts a glutamine residue from the small domain into the cavity, left by the flipped base. In the case of the M.HaeIII enzyme (another cytosine-C5 MTase), the orphan G pairs with a C on the 3′ side of the target base. The partner of this C is orphaned and the large cleft in the DNA is filled with solvent. In the case of the TaqI enzyme (an adenine-N6 MTase) the orphan T moves towards the center of the DNA helix. Thereby, in part, it occupies the space left by the flipped A, and in this way optimizes its base stacking interactions.

Base flipping is at least a two-step process. The base is first extruded from the DNA and then tightly contacted by the enzyme in a binding pocket in the catalytic domain allowing base recognition and catalysis to occur. Whereas the first step of the flipping process is very fast (milliseconds), the second is much slower (seconds). It is not clear how flipping of the target base is initiated; it could simply be spontaneous DNA breathing where thermal energy occasionally leads to the flipping of the base. The function of the enzyme would be to catch the flipped base and prevent its back-rotation until after methylation had occurred. Alternatively, contacts of the enzyme with the phosphodiester groups of the backbone of the DNA might impose torsional stress on the DNA that would induce the flipping. It is clear that the interactions of the enzyme with the target base itself are not required for base flipping, at least in the case of the HhaI MTase, because with this enzyme a rotation of the deoxyribose analogous to base flipping is also observed using substrates carrying an abasic site at the target position. Finally, we do not know whether base flipping is correlated with DNA recognition. In principle, two alternative reaction schemes are possible. The enzyme could scan the DNA, recognize its target base in the unflipped state, and then induce flipping only at the specific site. Alternatively, flipping could occur anywhere and the enzyme recognized the target site only after flipping. Then it would lock the flipped base only if the correct sequence is bound.

## Molecular Enzymology of DNA MTases

In this section, we shall briefly describe some features of DNA MTases, which explain how these fascinating enzymes are able to perform their complicated function.

### Structures of DNA MTases

Structures of six DNA MTases are presently available: M.HhaI, M.HaeIII, M.TaqI; M.PvuII, DpnM, M.MboII, and M.RsrI. Whereas for M.HhaI, M.HaeIII, and M.TaqI ternary complexes between cofactor, DNA and enzyme are solved, all other structures are binary complexes between the Mtase and different forms of the cofactor. The single present exception is the structure of a catalytically compromised mutant of M.RsrI, L72P, that lacks bound ligand. All DNA MTases analyzed so far comprise two domains, one large and one small.

#### Structure and Function of the Large Domain

The large domain consists of a central mixed $\beta$-sheet, flanked by $\alpha$-helices. The overall structure consists of two subdomains, which both form a hydrophobic pocket, the cofactor-binding site, and the binding site for the flipped target base. The AdoMet-binding site is composed of a subset of the conserved amino acid motifs that are found in most AdoMet-interacting enzymes, with conserved motif I ((D/E/S)XFXGXG) contributing important contacts to the AdoMet. Surprisingly, C-MTases and N-MTases show a striking similarity in the structure of their catalytic domains, with important catalytic residues (motif IV: PCQ in the case of cytosine-C5 MTases and (D/N/S)PP(Y/F) in the case of N-MTases) occupying equivalent positions in the three-dimensional structures of both MTase families. This finding was surprising, given the apparent differences in the catalytic mechanisms of both types of enzymes. It suggests an evolutionary relationship of both classes of MTases. In this context, it is interesting to note that three families of N-MTases exist that are related by circular permutations of the catalytic domain, but all known cytosine-C5 MTases belong to just one form that corresponds to the $\gamma$-family of N-MTases. Given the fact that N-MTases are structurally much more diverse and that they have a wider range of substrates (adenine, cytosine, and also amino acids), one might speculate that the C-MTases could be derived from the N-MTases, taking advantage of their established AdoMet-binding site and ability to flip the target base.

#### Structure and Function of the Small Domain

The second, smaller, domain of DNA MTases is much more heterogenous in size and structure. It contains most of the residues that mediate sequence-specific recognition of the DNA. Therefore, the variability of the target sequence is reflected by the loss of sequence conservation of the small DNA MTase domains. Upon specific DNA binding, base flipping is initiated or stabilized and conformational changes of the enzyme are induced. For example, in the case of M.HhaI, the loop comprising the catalytic motif IV of the enzyme undergoes a massive conformational change and approaches the DNA. Moreover, the small domain itself also moves towards the DNA. These changes are induced by the formation of base-specific contacts in a highly cooperative process that mediates the sequence specificity of DNA recognition. Thereby, the activation of the catalytic center is coupled to DNA recognition – a mechanism that is generally used by enzymes that interact with DNA in a sequence-specific manner.

### Mechanism of DNA Recognition

DNA recognition by MTases arises from sequence-specific contacts of the enzymes in the major and minor grooves of the DNA as well as to its phophodiester backbone. The small domain of the MTases contacts the

DNA from the major groove side, and the body of the large domain interacts with the minor groove. For example, M.HhaI contacts the edges of the bases of the recognition sequence with 14 hydrogen bonds. In addition, in M.HhaI and M.HaeIII, residues following the active-site motif IV are involved in DNA recognition and form base-contacts with the target sequence. A contribution of loops of the catalytic domain in sequence recognition was confirmed by biochemical experiments with M.SinI and M.EcoRII, which are both C-MTases, as well as with the adenine-N-MTase M.EcoRV.

Depending on the particular enzyme, specific-complex formation sometimes, but not always, leads to strong bending of the DNA. Unfortunately, structural information is available only for enzymes that do not induce bending of the DNA. It is interesting to note that the specificities of different DNA MTase can vary dramatically. Some MTases display a very high specificity that is comparable to that of restriction enzymes whereas others interact with the DNA in a more relaxed manner, such that sites differing from the consensus target site are modified at reasonable rates. Relaxed target specificity may arise because RM systems are steadily evolving and are regularly changing or modifying their recognition sites. Most likely, MTases with relaxed specificity have been caught in the act of molecular evolution and have not yet optimized selectivity for their new target site. The EcoRV MTase provides revealing details of this process. GATATC is the target site of this enzyme, and according to multiple sequence alignments, it is most closely related to dam MTases whose target site is GATC. Interaction with the same sequence expanded by two base pairs at the 3′ end suggests that EcoRV could have been derived from a GATC methylating enzyme. In support of this model, EcoRV also modifies GATC sites. The contacts of the EcoRV and dam enzymes to the GAT-part of their recognition sites are conserved in both enzyme families. The contacts to the ATC-part of the recognition site by EcoRV supplements the contacts to the conserved core GAT sequence. Interestingly, the contacts between M.EcoRV and the conserved core occur earlier in complex formation than those to the supplemental sites suggesting a pathway by which evolution could change the specificity of protein–DNA interaction.

## Mechanism of Target Site Location and Processivity of DNA Methylation

Ordinarily, DNA MTases methylate DNA only at specific sites. Localization of these specific sites in the sea of non-specific sites is a challenging process. Like most other proteins or enzymes that interact with DNA in a sequence-specific manner, DNA MTases make use of facilitated diffusion to accelerate the search for target sites. In this mechanism, the enzyme first binds to the DNA at a nonspecific site in a very fast reaction that often is close to the theoretical limit for a bimolecular association process. Then, the enzyme follows the DNA in a one-dimensional random movement.

One interesting facet in the target-acquisition mechanism of the DNA MTases is the manner in which several target sites are modified when they reside on the same DNA molecule. Enzymes can react with substrates containing several target sites in two principal ways: processively or distributively. In a processive reaction, after one turnover, the enzyme remains bound to the same substrate molecule, moves to the next target site and modifies it. Therefore, in a completely processive reaction, a DNA molecule is modified at all its target sites before the enzyme dissociates. In contrast, in a distributive reaction the enzyme releases the DNA after turnover at each site. As a consequence of these two mechanisms, the distribution of substrates, methylation intermediates, and products are completely different: methylation intermediates are not generated in a processive multiple-turnover reaction, whereas intermediates are necessarily formed in a distributive reaction. Recently, experiments with different MTases demonstrate that the solitary M.SssI, the CcrM MTase, and *E. coli* dam methylate DNA in a highly processive reaction. In contrast, the RM enzymes M.HhaI, M.HpaI, EcoRI, and EcoRV react distributively. This difference is particularly striking in the case of the EcoRV–*E. coli* dam pair, because they share high sequence similarity and show overlapping specificity.

Most likely, processive and distributive mechanisms are correlated with the biological roles played by the enzymes. One function of RM systems is to protect cells from bacteriophage infections, by cleavage of the phage DNA catalyzed by the restriction enzyme. The role of the MTase is to prevent cleavage of the host genome by methylation. However, the MTase must not modify the incoming DNA too rapidly or the phage DNA would be protected. The distributive reaction mechanism of the RM MTases would slow the complete methylation of any one molecule leaving it more vulnerable to restriction enzyme, because cleavage of one or a few sites is sufficient for inactivation of the phage. In agreement with this model, all RM MTases examined to date show a distributive reaction. However, mechanisms other than distributive ones such as compartmentation or tight control of the expression levels of both types of enzymes could also ensure proper RM MTase function. For the solitary MTases, no such evolutionary pressure against processive methylation exists, because rapid methylation of the chromosomal DNA after replication is desirable. For instance, prolonged hemimethylation of the newly replicated DNA, by delay of methylation

completion, would confuse the dam mismatch-repair system. Dam repair depends on the damage residing in the unmethylated strand, which holds true only for polymerase mistakes and not for other lesions in the DNA that might have occurred after replication.

The difference in processivity between EcoRV and *E. coli* dam is due to a pronounced difference in the order of DNA and AdoMet binding. Whereas EcoRV first binds to the cofactor and then to the DNA, *E. coli* dam prefers the opposite order of binding. Microscopic reversibility dictates that the order of DNA and cofactor release must reflect the order of binding. Thus, EcoRV first releases the DNA and then the cofactor. This mechanism precludes EcoRV from modifying DNA in a processive fashion, because it cannot exchange the product AdoHcy with AdoMet while bound to the DNA. What prevents an exchange of the cofactor in the ternary complex of M.EcoRV is presently unknown, but one could easily imagine that slight conformational changes of a few amino acid residues could open or block a channel that would permit cofactor exchange. Strikingly, subtle changes of the structure of the enzyme can be responsible for fundamental differences in the mechanism and thereby effect the adaptation of the enzymes to their biological functions.

## See Also the Following Articles

DNA Methyltransferases: Eubacterial GATC • DNA Mismatch Repair in Bacteria • DNA Replication: Initiation in Bacteria • DNA Restriction and Modification: Type I Enzymes • DNA Restriction and Modification: Type II Enzymes • DNA Restriction and Modification: Type III Enzymes

## Glossary

**base flipping** The process by which a base in the DNA is rotated so that it is no longer inside the DNA double helix, and is available for binding to an enzyme.

**epigenetic information** Genetic information arising from modifications of the bases in DNA.

**hemimethylation** Condition in which only one strand of the DNA double helix is methylated at a given site.

**nucleobase** One of the heterocyclic structures, adenine, cytosine, guanine, or thymine, that is attached to the phosphodiester-linked deoxyribose backbone of DNA.

**nucleophile** An atom or molecule group that is electron rich and reacts readily with a positively charged atom.

## Further Reading

Cheng, X. (1995). Structure and function of DNA methyltransferases. *Annu. Rev. Biophys. Biomol. Struct.* **24**, 293–318.

Cheng, X. (1995). DNA modification by methyltransferases. *Curr. Opin. Struct. Biol.* **5**, 4–10.

Cheng, X., and Blumenthal, R. M. (1999). *S-Adenosylmethionine-Dependent Methyltransferases: Structures and Functions*. World Scientific, Singapore.

Cheng, X., and Roberts, R. J. (2001). AdoMet-dependent methylation, DNA methyltransferases and base-flipping. *Nucl. Acids Res.* **29**, 3784–3795.

Jeltsch, A. (2002). Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. *Chem. Bio. Chem.* **3**, 274–293.

Klimasauskas, X., Kumar, S., Roberts, R. J., and Cheng, X. (1994). HhaI methyltransferase flips its target base out of the DNA helix. *Cell* **76**, 357–369.

Low, D. A., Weyand, N. J., and Mahan, M. J. (2001). Roles of DNA adenine methylation in regulating bacterial gene expression and virulence. *Infect Immun.* **69**, 7197–7204.

Reisenauer, A., Kahng, L. S., McCollum, S., and Shapiro, L. (1999). Bacterial DNA methylation: A cell cycle regulator? *J. Bacteriol.* **181**, 5135–5139.

Roberts, R. J., and Cheng, X. (1998). Base flipping. *Annu. Rev. Biochem.* **67**, 181–198.

## Biography

Albert Jeltsch is a Professor of Biochemistry at the School of Engineering and Science of the International University Bremen, Germany. He received his diploma from the University of Hannover, Germany and his doctoral degree from the Hannover Medical School, Germany. His research interests are DNA methylation and the molecular enzymology of DNA enzymes.

Richard I. Gumport is a Professor in the Department of Biochemistry and College of Medicine at the University of Illinois at Urbana-Champaign. He received his bachelor and doctoral degrees from the University of Chicago. His research interests are nucleic acid enzymology with an emphasis on protein–DNA interactions.

# DNA Methyltransferases, Structural Themes

**Sanjay Kumar**
*New England Biolabs, Beverly, Massachusetts, USA*

**DNA methyltransferases (MTases) are enzymes that catalyze the transfer of a methyl group from a donor molecule to DNA. On the basis of their chemistry, MTases can be grouped into two general categories: C5-MTases and N-MTases (Figure 1). The C5-MTases methylate the 5-carbon of the cytosine ring forming 5-methylcytosine. The N-MTases, which can be subclassed as N4- and N6-MTases, transfer a methyl group to an exocyclic nitrogen to form $N_4$-methylcytosine or $N_6$-methyladenine, respectively. The donor molecule in all known cases is the ubiquitous, small cofactor S-adenosyl-L-methionine (AdoMet or SAM). In the process of methylation, MTases employ a unique method called *base flipping* to access the target base. These enzymes are also highly selective DNA binding proteins and will only methylate their target bases within the context of a specifically recognized DNA sequence. MTases are widely distributed and have been isolated from or identified in the genomes of a wide variety of higher eukaryotes, lower eukaryotes, bacteria, and bacteriophage.**

## Function

The existence of DNA methylation (and hence MTases) has been known since 1948, even before the structure of DNA was discovered. Although much has been learned, the functional role of MTases in the molecular biology of the cell is still being uncovered today. DNA methylation is not essential for survival in all organisms, as both prokaryotes and eukaryotes that lack detectable methylation are known to exist. This is especially true in simpler organisms. However, as genome size increases (with some exceptions, of course), methylation takes on an increasingly important regulatory role, and defects in methylation are often strongly deleterious in plant and vertebrate embryonic development. The role of DNA methylation in human diseases is just beginning to be understood.

MTases impart epigenetic information to an organism's genome in the form of methylated bases. The primary purpose of this information is to modulate the interaction between DNA and the proteins that bind to it, whether directly by interacting with the proteins or indirectly by altering DNA structure. In general, methylation interferes with the binding of proteins to DNA; however, proteins whose DNA-binding is enhanced by methylation also exist. The effects of methylation on DNA structure are subtler. Methylation influences a variety of properties such as the stability of the double helix, DNA curvature, and the formation of DNA triple helices and left-handed Z-form DNA.

DNA methylation is closely tied to DNA replication. When a replication fork moves through a region of methylated DNA, the replicated DNA will be hemi-methylated with methyl groups *only* on the parental template strands. Soon afterward, the hemi-methylated DNA is recognized by MTases and becomes fully methylated once again. This transient undermethylated state can be used in a variety of ways: to monitor the replication state of DNA, to differentiate between the parental strand (methylated) and the newly synthesized strand (unmethylated), and to couple cellular functions to the cell cycle.

While MTases can aid in the correction of replication errors immediately after replication (called postreplicative mismatch repair) by using methylation to mark the strand carrying the original information, C5-MTases also contribute to the mutability of genomes. Cytosine spontaneously deaminates by hydrolysis at a very low rate, converting itself to uracil. Uracil is obviously foreign to DNA and the cell easily repairs the mutation. When 5-methylcytosine is deaminated it becomes thymine, giving rise to C → T transition mutations that are difficult for the cell to correct because T is a normal constituent of DNA. In addition, C5-MTases, in the presence of low concentrations of AdoMet, accelerate the rate of deamination. Sites of cytosine methylation are therefore mutagenic hotspots, and the genomes of higher eukaryotes such as vertebrates show significant depletion in the dinucleotide CG (the target sequence for vertebrate MTases).

### Prokaryotes

Prokaryotes have both C5-MTases and N-MTases (Figure 1). A significant characteristic of prokaryotic

 © 2004, Elsevier Inc. All Rights Reserved. 

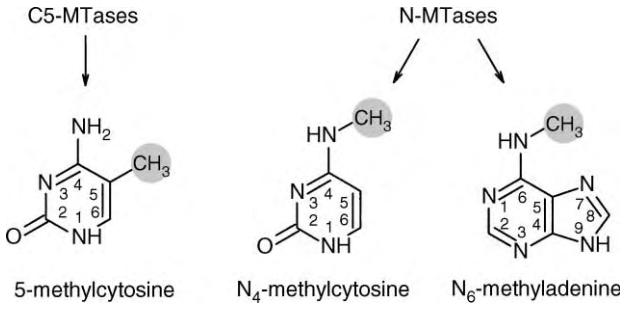



**FIGURE 1** Products of DNA methylation by MTases. A shaded gray circle highlights the added methyl group.

DNA methylation is its promiscuity: prokaryotic MTases will methylate all available target sites, and do not discriminate between hemi-methylated and unmethylated targets. DNA binding proteins may, however, delay this methylation at some sites by temporarily sequestering the target sequences. MTases in prokaryotes are involved in two major groups of functions: (1) restriction–modification (RM) systems that serve as immune defenses against phage infection and (2) regulation of cellular processes.

### RM Systems

In RM systems, MTases are paired with restriction endonucleases (RE). RE are enzymes that recognize target sequences in DNA with very high sequence specificity and then cleave the DNA. DNA methylation of the target sequence inhibits binding and cleavage by the RE. In an RM system, the MTase and RE have identical or overlapping target sequence specificity. The bacterium's DNA is protected from RE cleavage by prior methylation at the recognition site. Incoming foreign DNA (classically phage DNA), which does not display the same pattern of methylation as the host, is quickly fragmented by the host's REs. In this way, the bacterium uses MTases to effectively discriminate between self and foreign DNA.

### Regulation of Cellular Processes

Our knowledge of the regulation of prokaryotic cellular processes by methylation is almost entirely focused on the action of one MTase: the Dam MTase, a solitary N-MTase (not part of an RM system) that recognizes the sequence GATC and methylates the adenine residue. The primary signal used for regulation is the transient undermethylation of Dam sites after replication. Dam MTase is involved in postreplicative mismatch repair, as well as controlling aspects of the initiation of chromosomal and plasmid DNA replication, transposition of transposable elements, and gene expression.

## Eukaryotes

DNA methylation in eukaryotes is exclusively in the form of 5-methylcytosine and is confined to the dinucleotide CG (or CNG in plants, where N is any nucleotide), and C5-MTases are the only type of MTases found in eukaryotes. While little or no methylation is detected in unicellular, insect or invertebrate cells, both plant and vertebrate DNA are significantly methylated. Approximately 3–8% of the cytosine residues in vertebrates and 4–40% in plants are methylated. Unlike the prokaryotes, where the distribution of methylated bases mirrors the distribution of target sequences, only 60–80% of eukaryotic CG sites have methyl groups attached; however, for any given site, the cytosines in both strands of the DNA are methylated.

### Pattern of Methylation

While eukaryotic DNA methylation is incomplete, it is not random. The pattern of methylation can be tissue specific and maintainable across cell generations. This implies the existence of at least two types of MTase functions: a *de novo* function to establish methylation patterns and a maintenance function to faithfully copy those patterns following DNA replication. The maintenance methylase function, exemplified by mammalian DNMT1, preferentially targets the hemi-methylated sequences that are present after DNA replication, a preference not shared by bacterial MTases. The CG dinucleotide distribution is also nonrandom. In mammals, while CG is generally depleted, it occurs at its expected frequency in blocks of sequence called CpG islands. CpG islands are often located near sites that regulate the transcription of genes. While they contain only 15% of the genome's CG sites, over half the target CG sequences in CpG islands are unmethylated. In contrast, 80% of the remaining CG sites in the genome are methylated.

### Transcriptional Silencing

The effects of DNA cytosine-5 methylation in eukaryotes are complex and a thorough discussion is beyond the scope of this entry. However, there is strong evidence for its involvement in partitioning the genome into transcriptionally active and inactive functional compartments, where DNA methylation is generally correlated with silencing of gene expression. Methylation appears to accomplish this silencing by recruiting transcriptional repressors to the methylation sites, as well as histone deacetylases. The latter are involved in remodeling the chromatin structure that packages eukaryotic DNA into

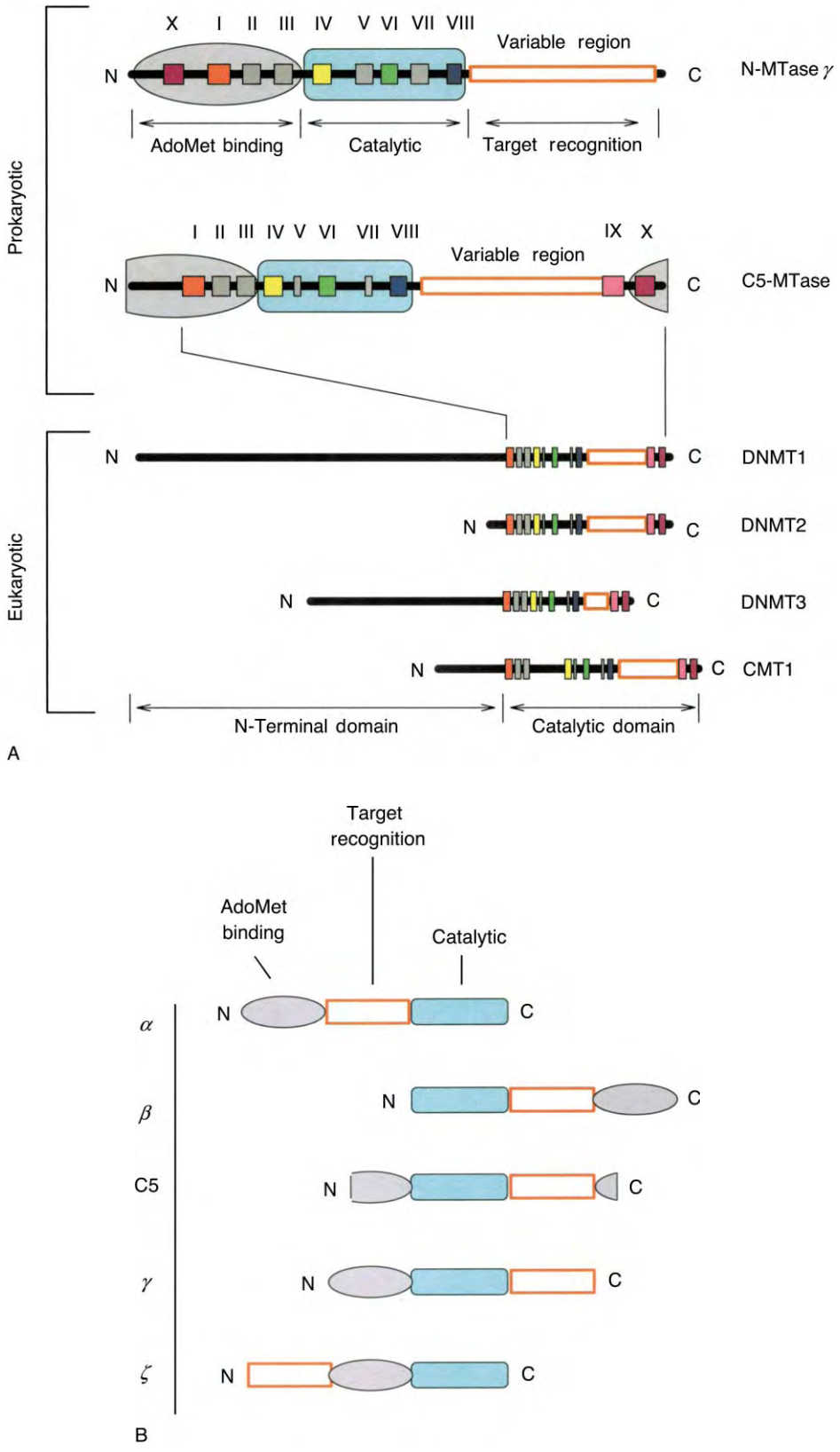



**FIGURE 2** Schematic of MTase motifs and domains. (A) Sequence motifs and modules: Motifs are numbered I through X (Roman numerals). Conserved motifs are consistently represented by the same color square in all figures (not to scale). The MTase structure is divided into three modules: AdoMet Binding (gray oval), Catalytic (cyan rectangle), and Target Recognition (thin brown rectangle). The Target Recognition module and the variable region overlap completely. The N-MTase schematic is specific for the $\gamma$ class of N-MTases. In C5-MTases, the components of the AdoMet Binding module are split and portions reside on the N and C termini of the sequence. The eukaryotic enzyme families (DNMT1, 2, 3,

a transcriptionally silent state. Methylation induced transcriptional silencing is believed to play a role in such processes as embryonic development, genomic imprinting, X-chromosome inactivation, and silencing of transposons and other mobile repetitive elements.

## Primary Sequence Organization

### Modular Arrangement

MTases are built from three basic modules that perform the primary functions required of these enzymes: DNA recognition, AdoMet binding and catalysis of methylation (Figure 2A). With few exceptions, they are monomeric proteins that range in size from approximately 300–500 amino acids in prokaryotes to over 1500 amino acids in eukaryotes. The amino acid sequence of MTases is divided into two distinct regions. The first region contains many sequence motifs that are conserved between groups of MTases. The core enzymatic functionality, consisting of the AdoMet binding and catalytic domains, is located in this region. The other region, termed the "variable region" (because its size and sequence varies greatly between otherwise related MTases), is almost completely devoid of such motifs (Figure 2A). The variable region contains the target recognition domain (TRD) and forms the third basic module. Theoretically, six permutations of the linear order of these three modules are possible (Figure 2B). To date, examples of four of the six have been identified.

### Conserved Sequence Motifs

The signatures of their sequence motifs can often identify different classes of MTases. C5-MTases share a set of up to 10 motifs (labeled I–X in Figure 2A), six of which are strongly conserved. Each motif spans a total of 8–20 amino acids and usually contains two or three highly conserved positions. The linear order of these motifs is also conserved in virtually every member of the C5-MTase family, and the variable region is always located between motifs VIII and IX. The 10 motifs are also found in eukaryotic MTases, but they are confined to the roughly 500 amino acid long C-terminal catalytic domain of these larger enzymes. The eukaryotic MTases often possess an additional very large N-terminal domain that modulates their biological function.

Nine motifs can be located within N-MTases at positions that spatially overlap their C5-MTase counterparts on protein structures (Figure 2A); however, the level of similarity at the primary sequence level between corresponding C5 and N-MTase motifs is very low. Even corresponding motifs in different groups of N-MTases can show substantial differences. Only two of the sequence motifs, motif I and motif IV, can be identified without the aid of protein structure information. The N-MTases can be grouped into subclasses on the basis of motif order, but these groupings do not distinguish between $N_4$-cytosine and $N_6$-adenine MTases. The variable region is inserted in different, but conserved, positions relative to the motifs in each group.

### Tertiary Structure

Reflecting the modularity seen in the primary sequences, MTases fold into structures with two domains: the core methylation machinery is localized in one large domain and the sequence recognition functions are concentrated in a second usually smaller domain. DNA binds in the cleft between the two domains, which are connected by a stalk or hinge (Figures 3 and 4).

#### Large Domain

The large domain is dominated by a structural motif termed the AdoMet-dependent methyltransferase fold that is conserved across a wide variety of AdoMet-dependent methyltransferases. The AdoMet fold spans both the AdoMet binding module and the catalytic module (Figure 3). It is composed of a core seven-stranded $\beta$ sheet with strand order 6↓, 7↑, 5↓, 4↓, 1↓, 2↓, 3↓ (the arrows indicate relative strand direction), though strand 3 is sometimes not well formed. A characteristic feature is the insertion of strand 7 between strands 6 and 5 and antiparallel to the other six strands. The topological switch point (where the strand order changes) between strands 4 and 1 is situated in the active site. The switch point roughly divides the AdoMet fold into two parts, with strands 1 to 3 forming the core of the AdoMet binding module and strands 4 to 7 forming parts of the catalytic module including the substrate binding pocket. The two sections of the AdoMet fold $\beta$-sheet are each flanked by $\alpha$ helices to form $\beta$-$\alpha$-$\beta$-$\alpha$ sandwiches. The core structure of the large domain is structurally quite similar to the Rossman fold of proteins that bind NAD or NADP.

#### Small Domain

The small domain corresponds to the "variable region" in primary sequence alignments of MTases.

and CMT1) all share the same motif arrangement as the prokaryotic C5-MTases, but the region of similarity is entirely confined to the C-terminal catalytic domain of these larger enzymes. (B) The modular arrangement of known MTases: The $\alpha$, $\beta$, $\gamma$, and $\zeta$ classes are exclusively N-MTases. The C5 class (C5-MTases) and the (N-MTases are essentially the same class. The arrangements of domains in the $\beta$, C5, $\gamma$, and $\zeta$ classes are circular permutations of each other.

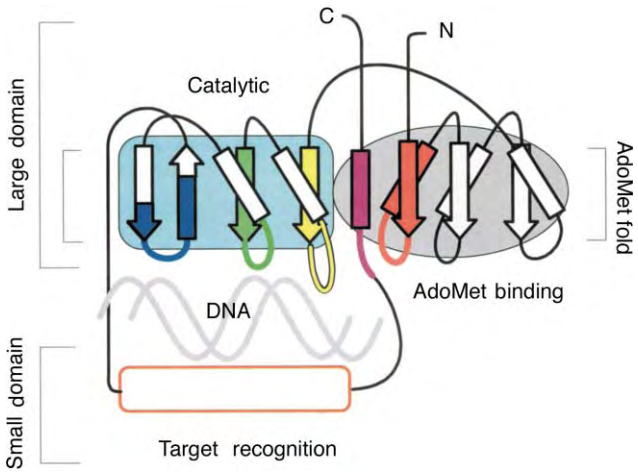



**FIGURE 3** Structure of MTases: An idealized representation of the structure of a C5-MTase is shown. $\beta$ strands are represented by arrows, $\alpha$ helices by rectangles, and loops by curved lines. Gray wavy double lines indicate DNA. Colored regions represent the locations of sequence motifs (Figure 2A). Other symbols and colors represent the same as those described in Figure 2. $\beta$ strands are numbered 6, 7, 5, 4, 1, 2, and 3, from left to right as shown.

Not surprisingly, this domain is varied in size and structural arrangement. The small domain contacts the major groove face of the target DNA. In C5-MTases, the residues that specify the choice of target sequence and the base to be methylated generally reside on the small domain. In N-MTases, the contacts are more distributed between the two DNA grooves and the two domains of the enzyme.

## Mechanism

### Chemistry

The methylation reaction catalyzed by MTases requires only the DNA substrate and AdoMet. The products of the reaction are the various methylated bases and S-adenosyl-L-homocysteine (AdoHcy or SAH). The methylated bases are relatively stable, and the reverse reaction (demethylation) is not known to be mediated by MTases.

#### AdoMet

AdoMet is the most commonly used methyl donor in cellular biochemistry and the second most common

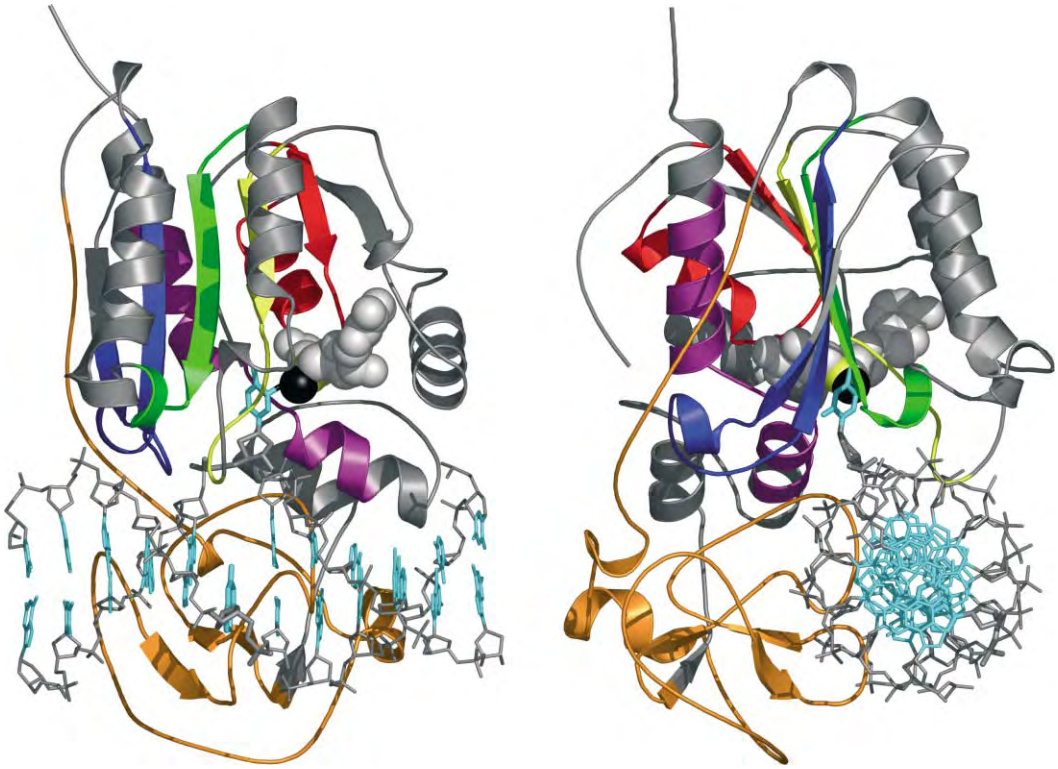


**FIGURE 4** Crystallographic Structure of a C5-MTase: A three-dimensional cartoon representation of the structure of the C5-MTase M.HhaI. $\beta$ strands are represented by arrows, $\alpha$ helices by helical flat coils, and loops by thin, curved lines. Protein is gray. The variable region is shown in brown. Other colors represent the same as those in Figures 2 and 3. DNA is seen in the lower part of the structure, with a gray sugar-phosphate backbone and cyan bases. The target base near the center of the structure is flipped out. The small gray molecule represented by space-filling spheres is AdoMet. The carbon to be transferred is colored black. The two views are related by a 90° rotation around the vertical axis of the structure.

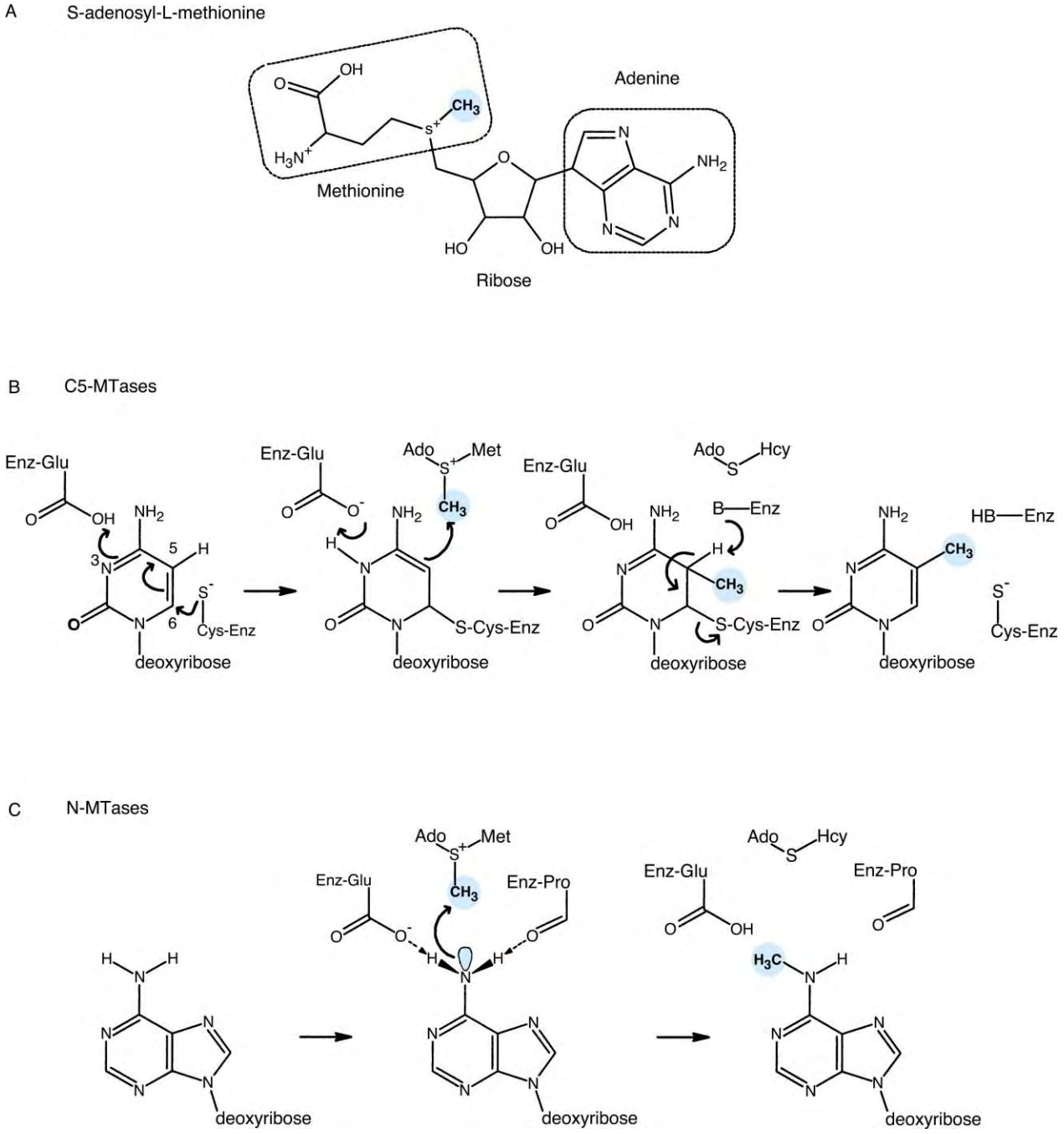



**FIGURE 5** Reaction mechanisms of MTases: In each panel, a shaded gray circle indicates the transferred methyl group. The enzyme is represented by the symbol Enz. The symbol B refers to a generic basic residue. In (B) and (C), AdoMet is represented by the symbol Ado-S-Met and AdoHcy by Ado-S-Hcy. (A) The structure of S-adenosyl-L-methionine (AdoMet or SAM). (B) C5-MTase reaction mechanism. The mechanism is described in the text. Though not obvious from the diagram, the mechanism requires that the sulfhydryl group of Cys-Enz and the methyl group of AdoMet must approach perpendicular to and on opposite faces of the target cytosine. (C) N-MTase reaction mechanism based on the M.Taq I MTase. In the central structure, the coordinating hydrogen bonds (shown by dashed lines) are situated in a tetrahedral arrangement relative to the nitrogen and change the hybridization state of the nitrogen from $sp^2$ to $sp^3$. This localizes and repositions the free lone pair electrons, shown as a shaded orbital above the nitrogen, for nucleophylic attack on the methyl group.

cofactor after ATP (Figure 5A). The transferable methyl group resides on a charged sulfur atom, making it very reactive to nucleophiles such as polarized N, S, O, and activated carbons or carbanions. Interestingly, the synthesis reaction for AdoMet is the only one known to require hydrolysis of all three phosphates of ATP to drive the reaction. Transfer of the methyl group converts AdoMet to AdoHcy.

### C5-MTases

C5-MTases transfer a methyl group to the 5-carbon of the cytosine base (Figure 5B). The 5-carbon of cytosine is not normally reactive enough to attack the methyl group on AdoMet to effect transfer. C5-MTases catalyze this unfavorable reaction in the following way, with a key feature of this process being the formation of a transient covalent complex between the enzyme and the DNA. First, a cysteine thiol on the enzyme serves as a nucleophile that attacks the 6-carbon of the pyrimidine ring, forming a covalent DNA-protein intermediate. This is aided by the protonation of N3 on the ring by the enzyme. The sulfhydryl is provided by the Cys in the highly conserved PC dipeptide (motif IV) found in all C5-MTases. A Glu in the highly conserved ENV tripeptide (motif VI) carries out the protonation. An Arg residue from motif VIII assists the process. This addition activates the 5-carbon and allows it to attack the AdoMet methyl group. Following transfer, AdoHcy is released. The proton left at the 5-position is abstracted by a nearby basic residue leading to resolution of the DNA-protein complex by $\beta$-elimination.

### N-MTases

$N_4$-cytosine and $N_6$-adenine N-MTases methylate an exocyclic nitrogen atom (Figure 5C). Their mechanisms are believed to be similar, but they are not as clearly understood as for the C5-MTases. Methylation occurs by direct transfer from AdoMet and does not involve a covalent DNA-protein intermediate. Each of the first two residues of conserved motif IV (N/S/DPPY/F) donates a hydrogen bond to the target exocyclic nitrogen. This activates the exocyclic N for subsequent nucleophylic attack on the AdoMet methyl group. Following transfer, AdoHcy is released.

## AdoMet and DNA Binding

### Association of the Reactants

Methylation reactions proceed via a complex between the MTase, DNA, and AdoMet. The order of association of these components varies among MTases (sometimes being random) and no general rule applies to all MTases. The binding to DNA occurs in two discrete steps. The initial nonspecific DNA binding is accompanied by scanning linear diffusion, in which the MTase slides along the DNA in search of a specific recognition site. In the second step, the MTase binds tightly to a specific recognition sequence. When specific binding is observed, it is often associated with a substantial conformational change in the DNA and/or the MTase.

### Sequence-Specific DNA Recognition

Sequence-specific recognition of DNA is achieved through multiple contacts between the MTase and the target DNA, both direct and water-mediated. The target base itself does not seem to be part of the recognition sequence as DNA sequences with missing, modified, or alternate target bases are still recognized by the appropriate MTase. In fact, several MTases show preferential affinity for DNA substrates containing base mismatches at the target base position. The vast majority of MTases recognizes palindromic sequences and thus methylates both strands of a target sequence.

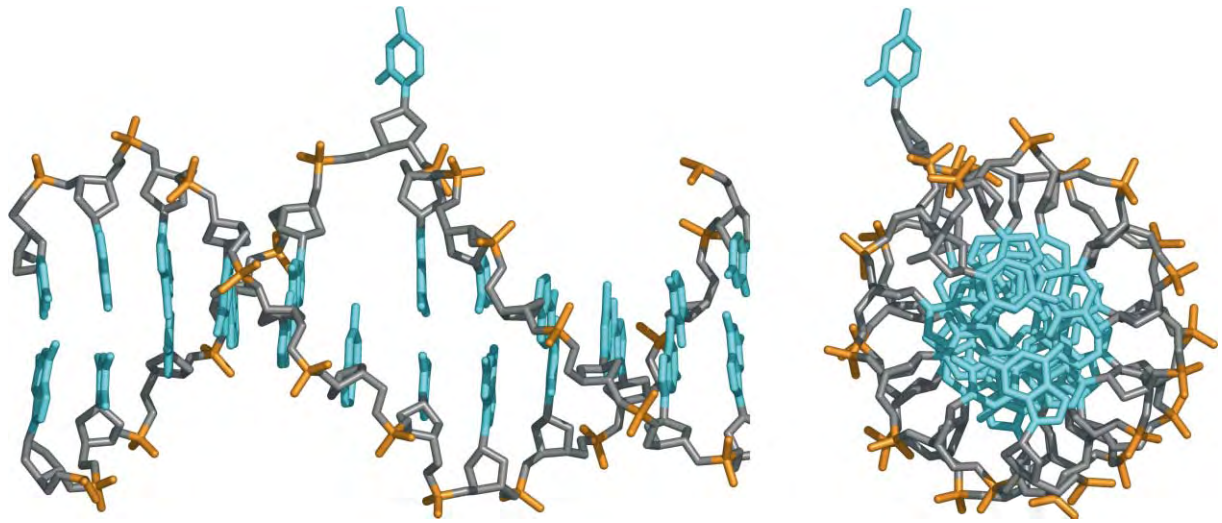


**FIGURE 6** DNA base flipping: The DNA (shown from the side and end-on) is in the same orientation as in the structure shown in Figure 4. The backbone is gray with orange phosphates and the bases are cyan. With the exception of the flipped cytosine, the structure is classical B-Form DNA.

## Base Flipping

The target base for MTases is normally buried in the DNA helix stack, a location that is incompatible with the stereochemical requirements of the reacting groups on the cofactor and the enzyme. The MTases overcome this spatial hurdle in a surprising and elegant manner: without seriously distorting the remainder of the DNA helix or breaking any bonds, they rotate the target base and its sugar phosphate backbone 180° out of the helix stack and into a pocket in the active site (Figures 4 and 6). This process has been termed "base flipping." The precise mechanism of base flipping is unknown. However, the identity of the target base or even its presence appears irrelevant to the mechanism, as mismatched, modified, methylated, or even missing bases at the target site still display base-flipping, or flipping of the remaining sugar-phosphate backbone at the target position. Subsequent to its discovery in MTases, base flipping was shown to be utilized by other proteins that need access to bases buried within DNA, including DNA glycosylases and AP endonucleases.

## See Also the Following Articles

Chromatin Remodeling • Chromatin: Physical Organization • DNA Methyltransferases, Bacterial • DNA Methyltransferases: Eubacterial GATC • Nuclear Organization, Chromatin Structure, and Gene Silencing • Transcriptional Silencing

## Glossary

**beta sheet** A flat, side-by-side arrangement of polypeptide chains linked together by hydrogen bonds. Each chain is in a zigzag conformation called a beta strand. Beta strands that run in the same direction (from amino terminal to carboxyl terminal) are termed parallel, while those that run in opposite directions are termed antiparallel.

**chromatin** DNA packaged around small protein cores. Roughly 200 base pairs of DNA are wound twice around an octamer of proteins called histones to form a nucleosome. Nucleosomes are the basic repeating structure of chromatin in eukaryotes.

**CpG island** A short region of DNA (one kilobase or less in length) that contains clusters of CG dinucleotides (the p in CpG refers to the intervening phosphodiester) at a frequency that is expected from the base composition of the DNA. CpG islands are often located in proximity to the transcriptional control regions of genes. They are normally undermethylated.

**hemi-methylated** Referring to a double-stranded DNA segment containing a recognition sequence for a MTase in which only one strand of the target sequence is methylated.

**palindromic sequence** A DNA sequence in which the sequence and its complement are identical when each is read in the standard 5′ to 3′ direction. CG, GATC, and GAATTC are examples of palindromic sequences.

## Further Reading

Alberts, B., Lewis, M., Raff, M., Johnson, A., and Roberts, K. (2002). *Molecular Biology of the Cell*. Taylor & Francis, New York.

Cheng, X., and Blumenthal, M. (eds.) (1999). *S-Adenosylmethionine-Dependent Methyltransferases: Structures and Functions*. World Scientific, New Jersey.

Cheng, X., and Roberts, R. J. (2001). AdoMet-dependent methylation, DNA methyltransferases and base flipping. *Nucl. Acids Res.* **29**, 3784–3795.

Jeltsch, A. (2002). Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. *Chem. Bio. Chem.* **3**, 275–293.

Jones, P. A., and Takai, D. (2001). The role of DNA methylation in mammalian epigenetics. *Science* **293**, 1068–1070.

Jost, J. P., and Saluz, H. P. (eds.) (1993). *DNA Methylation: Molecular Biology and Biological Significance*. Birkhäuser Verlag, Basel.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J., and Wilson, G. G. (1994). The DNA (cytosine-5) methyltransferases. *Nucl. Acids Res.* **22**, 1–10.

Robertson, K., and Wolffe, A. P. (2000). DNA methylation in health and disease. *Nat. Rev. Genet.* **1**, 11–19.

## Biography

Sanjay Kumar is a Staff Scientist at New England Biolabs in Beverly, Massachusetts. His research interests include DNA methyltransferases, DNA and chromatin structure, and the development of bioinformatic tools. He holds a Ph.D. in biomedical sciences from Wright State University in Dayton, Ohio. He received his postdoctoral training with Dr. Richard Roberts at Cold Spring Harbor Lab in Cold Spring Harbor, New York. Dr. Kumar and his associates authored the first paper to detail the crystallographic structure of a DNA methyltransferase, and later they made the first discovery of base flipping in DNA.

# DNA Methyltransferases: Eubacterial GATC

**Martin G. Marinus**
*University of Massachusetts Medical School, Worcester, Massachusetts, USA*

**Eubacterial GATC methyltransferases transfer methyl groups from S-adenosyl-L-methionine to the N-6 position of the adenine ring. Only adenines in the tetranucleotide sequence GATC in double-stranded DNA are methylated. Unlike other methyltransferases that function to protect DNA from restriction endonucleases, the eubacterial GATC methyltransferases have other biological functions that are described in this article. In general, the cell uses DNA methylation primarily to control the rate at which these functions exert their effects. Most of our knowledge about eubacterial GATC methyltransferases has come from studies using the Dam methyltransferase of *Escherichia coli* as a model system and this article will focus primarily on it.**

## Properties of Dam Methyltransferase

The Dam methyltransferase is encoded by the *dam* (*D*NA *a*denine *m*ethyltransferase) gene of *Escherichia coli*, which has a complex mechanism of gene regulation involving multiple promoters and terminators. The major promoter for transcription of the gene is regulated by growth rate of the cells; that is, the faster the growth rate the greater the level of initiation of transcription.

During chromosome replication, the replication fork is followed by a region of hemimethylated DNA which comprises new unmethylated DNA and the complementary methylated template strand. After ~1 min, on average, the newly synthesized DNA is methylated so that both strands are fully methylated. This delay in methylation is due to the concentration of the Dam methyltransferase; it is present at ~130 molecules per cell. Increasing the cellular concentration of the enzyme decreases proportionately the amount of hemimethylated DNA.

The enzyme is a single polypeptide chain of 278 amino acids with a molecular weight of 32 kDa and exists in solution as a monomer. The enzyme has a turnover number of 19 methyl transfers per min and an apparent $K_m$ of 3.6 nM for DNA and double-stranded DNA is a better methyl acceptor than denatured DNA and there is one methyl transfer per site per binding event even if the substrate DNA is fully unmethylated. The protein may have two SAM binding sites: a catalytic site and one which increases specific binding to DNA perhaps as a result of an allosteric change. The methyltransferase is thought to bind the template and slide processively along the DNA, methylating about 50 sites before dissociating.

## Properties and Uses of *∂am* Mutant Strains

The commonly used *dam* mutant strains do not have detectable residual methyltransferase activity nor detectable methylation at GATC sequences. *Dam* mutants are most often used to "launder" DNA molecules that have sites that are resistant to digestion with specific restriction endonucleases. These include: AlwI, BcgI, BclI, BsaBI, BspDI, BspEI, BspHI, ClaI, HphI, NruI, TaqI, and XbaI. Resistance occurs because these enzymes have recognition sequences that overlap with the GATC tetranucleotide and almost all of these are methylated in wildtype *E. coli*. After transfer through the *dam* mutant strain, there is no methylation at GATCs and DNA molecules are now sensitive to digestion with the restriction endonucleases listed above.

*Dam* mutants have also been useful to define the biological roles of methyl groups on adenine in DNA. The mutant strain shows a large number of phenotypic differences compared to wild type suggesting multiple functions. All the known mutant phenotypes in *E. coli* can be explained by the involvement of GATC methylation in mismatch repair, regulation of gene expression, and initiation of chromosome replication.

## Dam-Directed Mismatch Repair

During chromosome replication, errors are made at low frequency by the replicative polymerase to form base

 © 2004, Elsevier Inc. All Rights Reserved. 

mismatches in newly synthesized DNA. Such errors need to be removed because they are potentially mutagenic. *E. coli* and most other organisms, including humans, have a highly conserved repair system that removes such mismatches in newly synthesized DNA. The Dam- (or methyl-) directed mismatch repair system in *E. coli* uses only hemimethylated DNA as a substrate thereby restricting its activity to the region of the chromosome immediately trailing the replication fork. The repair system is outlined in Figure 1. Briefly, base mismatches in hemimethylated DNA are recognized by the MutS protein followed by formation of a ternary complex with two additional proteins, MutL and MutH. MutH is an endonuclease active on hemimethylated GATC sites but only when complexed with the other Mut proteins on DNA. Following DNA incision, the UvrD helicase unwinds DNA in either the 5′ to 3′ direction or the reverse. The directionality is dependent on the location of the mismatch relative to the nearest GATC sequence. The unwound nicked DNA strand is digested by one or more exonucleases followed by resynthesis using the replicative polymerase. DNA ligation of the nick at the end of replication completes the process of repair and the hemimethylated DNA is eventually methylated.

Three lines of genetic evidence indicate the importance of DNA methylation in the process. First, overproduction of Dam methyltransferase inhibits the repair process by reducing the amount of hemimethylated DNA. Under these conditions mismatches are not repaired and lead to an increase in mutation frequency. Second, in a *dam* mutant the repair system is active but cannot discriminate the template strand from the daughter strand.

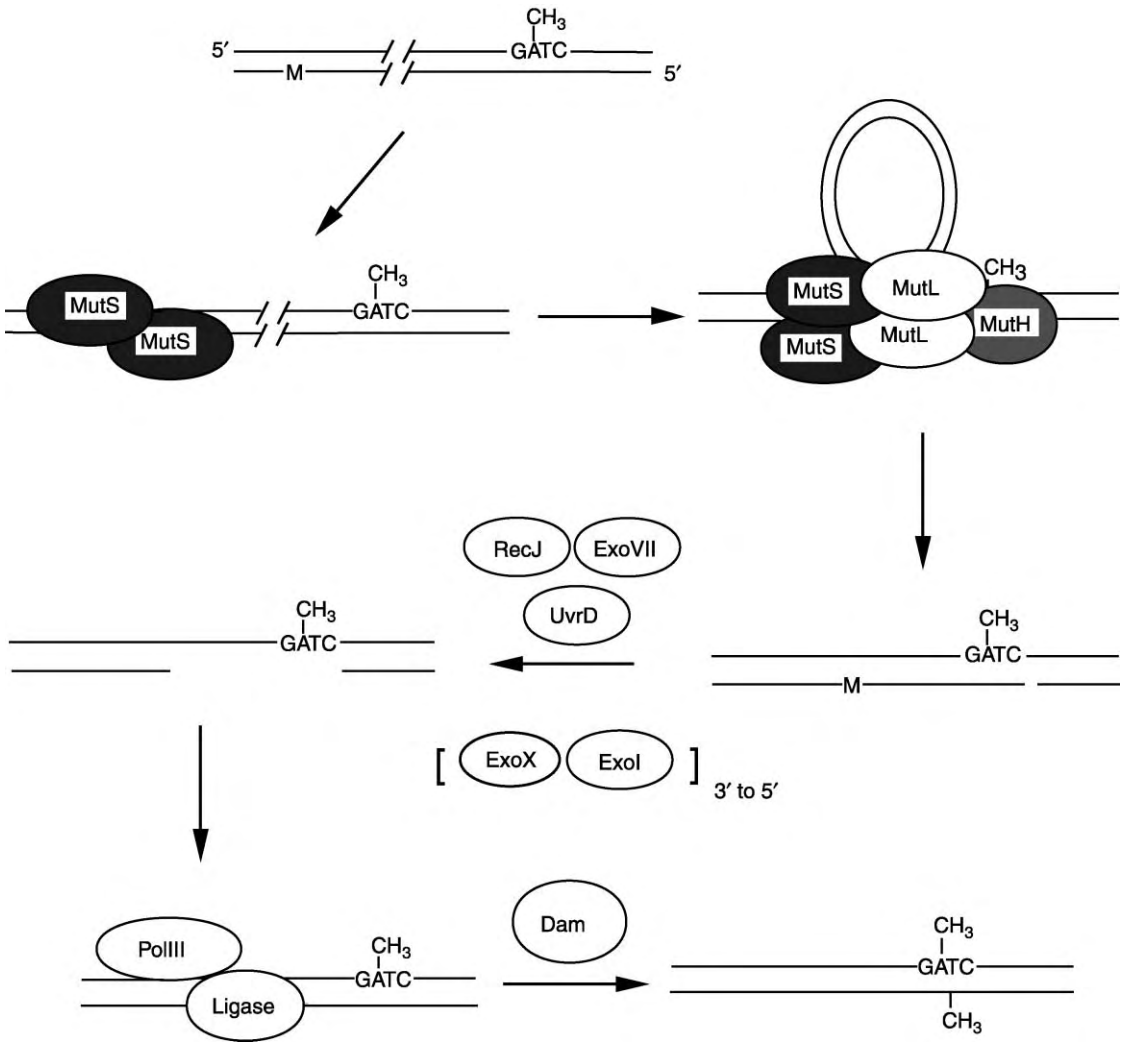



**FIGURE 1** DNA mismatch repair. A base mismatch (M) in the newly synthesized unmethylated DNA strand is some distance from the nearest GATC sequence. MutS protein binds the mismatch and a complex with MutL and MutH is formed leading to cleavage of the unmethylated strand. The UvrD helicase unwinds the unmethylated strand which is digested by an exonuclease. Excision can occur in either the 3′ to 5′ direction (ExoI, ExoX) or the reverse (RecJ, ExoVII) depending on the orientation of the mismatch relative to the GATC sequence. DNA polymerase III holoenzyme synthesizes a new strand and the resultant nick is ligated by DNA ligase. The GATC sequence is eventually methylated by Dam methyltransferase.

Consequently, the repair system removes mutations from the newly synthesized strand but also introduces mutations into the parental strand yielding an increased mutation frequency. Third, DNA duplexes containing a mismatch can be constructed with no methylation, methylation on both strands, or on only one of the two strands. When introduced into cells the fully methylated DNA is not repaired, hemimethylated DNA is repaired using the methylated strand as template and the unmethylated duplex is repaired using either strand as template.

In a *dam* mutant, mismatch repair produces detectable nicks or gaps in DNA but it is not known if the nicks or gaps occur exclusively behind the replication fork as a result of replication errors or in any part of the chromosome as a result of mismatches formed spontaneously. The nicks or gaps are converted to double-strand breaks either by a replication fork encountering an unrepaired gap or by activated MutH cleavage on complementary strands at the same GATC sequence or by a combination of these (Figure 2). It is not yet known which of these mechanisms (or both) generates double-strand breaks *in vivo* but *in vitro* evidence supports the MutH cleavage model.

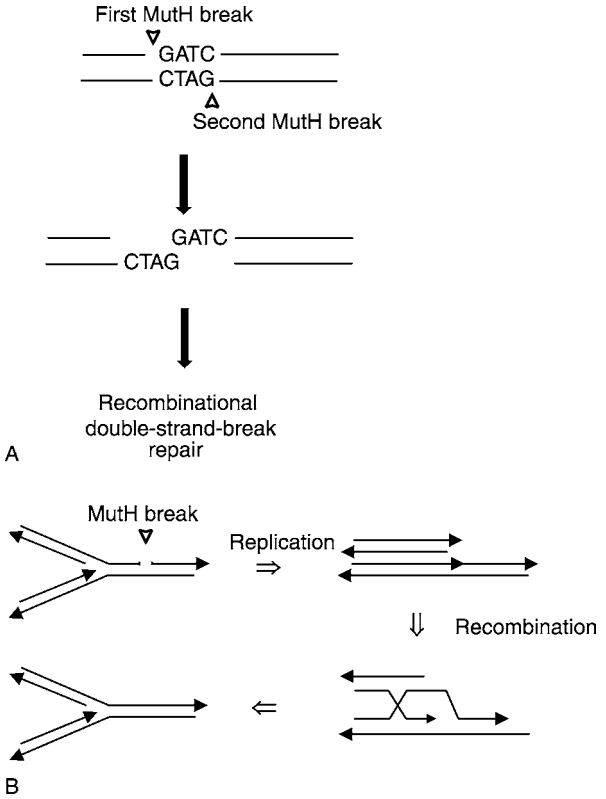



**FIGURE 2** Generation of double-strand DNA breaks. (A) Mismatch repair endonuclease MutH introduces nicks on either side of a GATC sequence to produce a double-strand break. (B) Mismatch repair introduces a nick ahead of a replication fork producing a double-strand break when the fork encounters it. Recombination is required to restore the fork.

Mismatch repair in *dam* mutants is also the basis for the phenomenon of drug tolerance. The mutants are more sensitive than wild type to certain anticancer agents such as cisplatin, and methylating agents like streptozotocin. Inactivation of mismatch repair, however, renders the cell tolerant to these agents. In other words, mismatch repair sensitizes *dam* cells to the effects of these deleterious agents. This is of interest because mammalian cells in culture respond in exactly the same way; that is, they are sensitive to these agents but become tolerant upon loss of mismatch repair capacity. The molecular basis for this phenomenon has yet to be defined.

## Initiation of Chromosome Replication

In *E. coli*, chromosome replication is initiated only once per cell cycle and Dam methylation is involved in this process. Chromosome replication is initiated at *oriC*, a region which has a 10 times higher than expected density of GATC sequences, by the initiation protein DnaA. The promoter region of the *dnaA* gene must be methylated for maximal expression and methylated origins are more efficiently initiated than unmethylated ones. The neighboring hemimethylated *oriC* and *dnaA* gene region is bound specifically and with high affinity by the SeqA protein which prevents methylation by Dam methyltransferase and renders the origin inert to further initiation (the eclipse period). By a process that is not understood, SeqA is released from *oriC* later in the cell cycle and methylation ensues followed by another initiation event.

Fast-growing wild type cells contain multiple origins and these initiate synchronously. The eclipse period is dependent on the level of Dam methyltransferase and can be lengthened or shortened by decreasing or increasing the enzyme level respectively. *In vivo*, *dam* and *seqA* mutants have an asynchronous initiation pattern and the eclipse period is shortened or absent. The *seqA* mutant, as expected, performs extra initiations each cell cycle but the *dam* mutant does not. This indicates that in *dam* mutants there must be an alternative mechanism preventing excessive initiation.

## Altered Gene Expression

The state of GATC methylation can affect protein–DNA interaction and if this tetranucleotide sequence occurs in promoter or regulatory DNA regions, gene expression can be altered. As discussed, the *dnaA* gene is maximally transcribed only if the promoter region is fully methylated. This is consistent with its role in the initiation of chromosome replication. Another example is the

promoter for the *tsp* (transposase) gene of the mobile genetic element, *Tn*10, which controls the rate of transposition. This promoter is active only when it is hemimethylated thereby ensuring that transposition is coupled with chromosome duplication and occurs only once per cell cycle. A further example is the *pap* (pyelonephritis-associated pilus) operon in pathogenic *E. coli*, where Dam and the transcriptional activator Lrp compete for binding to specific symmetrically unmethylated GATC sequences. Depending on which protein is bound, *pap* expression is either off or on.

There are few other genes where the rationale of coupling methylation to gene expression is so clear-cut. High-density oligonucleotide array analysis of mRNA from *dam* mutants indicates that expression of many genes is altered. In several instances this is due to competition between Dam methyltransferase and transcriptional activators such as Fnr and Cap competing for the GATC sites that overlap their recognition sequences. For other genes such as *sulA* (involved in control of cell division) and *glnS* (glutamyl-tRNA synthetase), which have GATCs in their promoter regions, expression is increased when they are unmethylated. The biological rationale for this is unclear especially since in a wild type background these promoter regions would never be unmethylated. There are also many examples where GATCs in the promoter region have no effect on initiation of transcription. At present, there are no rules to predict if a GATC sequence in the promoter will increase, decrease, or have no effect on the frequency of transcription initiation.

As discussed, *dam* mutant DNA is subjected to DNA breakage *in vivo*. This results in the induction of a stress response termed SOS in which transcription of about 50 genes is increased. The gene products include those that repair DNA damage, recombination, and control cell division. In *dam* mutants, this response is constitutively expressed because three recombination proteins (RecA, RuvA, and RuvB), which are part of the response, are required for cell viability.

## Role of Dam Methylation in Bacterial Pathogenesis

Altered levels of Dam methyltransferase can modulate the virulence of certain bacterial pathogens including *Salmonella*, *Yersinia*, and *Vibrio* in animal models and in *Erwinia*, a plant pathogen. The best-studied system is the *Salmonella*-mouse model. *Dam* mutant *Salmonella* are 10 000 times less capable of killing mice than wild type but a low level of tissue infectivity occurs. Mice that have been infected with the avirulent *dam* mutant become much more resistant to killing by wild-type *Salmonella* suggestive of a vaccine effect. The molecular basis for the role of Dam methylation in bacterial pathogenesis has yet to be elucidated but mismatch repair-deficient *Salmonella* are fully virulent. As there is a large group of virulence genes in *E. coli* and *Salmonella* whose expression is altered in *dam* mutants, it is likely that the basis for the avirulent phenotype of *dam* mutants is due to gene expression at inappropriate times allowing the animal hosts to mount an effective challenge to bacterial invasion.

A contrast to the *Salmonella* example is provided by *Neisseria* that can cause meningitis. These bacteria have a *dam* gene but it is not involved in pathogenesis. Rather, it is defects in the mismatch repair system (*mutS* and *mutL* gene inactivation) that allows for frameshift mutations to occur in DNA sequences controlling expression of certain virulence genes. There is no general rule about the effect of Dam methylation on virulence; it has to be investigated for each organism.

## Role of Dam Methylation in Other Bacteria and Their Viruses

At present, little is known about the role of Dam methylation in other bacteria. Inactivation of the *dam* gene in *Yersinia* and *Vibrio*, however, is a lethal event, although the reason is not known. In contrast, inactivation of the *dam* gene in *Neisseria meningitidis* is not lethal, does not lead to a mutator phenotype and inactivation of recombination does not lead to lethality. This suggests a fundamentally different role for Dam methyltransferase in this species compared to the *E. coli* paradigm.

The bacterial virus P1 has its own *dam* gene that is related to that of its *E. coli* host and is essential for its life cycle. Dam methylation is required at the stage where the viral DNA is packaged into the heads. This is accomplished by filling the head with genomic DNA and then cleaving it at specific sequences, termed *pac* sites. These *pac* sites are flanked by multiple GATC sequences which must be methylated in order for cleavage to occur. In other *E. coli* bacterial viruses, however, such as T2 and T4, deletion of the *dam* gene has no discernable effect on their life cycle. Again, no generalizations can be made regarding the essentiality of the *dam* gene for viability.

### See Also the Following Articles

DNA Methyltransferases, Structural Themes • DNA Mismatch Repair in Bacteria • Exonucleases, Bacterial

## Glossary

**DNA methyltransferase** Enzyme that transfers a methyl group to a base in DNA.

**epigenetic** Pertaining to phenotypic, but not genotypic, change.

**exonuclease** Enzyme that digests DNA strand from an end.

**hemimethylated** Describing DNA in which one strand contains methylated residues but the complementary strand does not.

**promoter** Sequence at which RNA polymerase initiates transcription.

## Further Reading

Casadesus, J., and Torreblanca, J. (1996). Methylation-related epigenetic signals in bacterial DNA. In *Epigenetic Mechanisms of Gene Regulation* (V. E. A. Russo, R. A. Martienssen and A. D. Riggs, eds.) pp. 141–153. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Cheng, X., and Roberts, R. J. (2001). Ado-Met-dependent methylation, DNA methyltransferases and base flipping. *Nucleic Acids Res.* **29**, 3784–3795.

Low, D. A., Weyand, N. J., and Mahan, M. J. (2001). Roles of DNA adenine methylation in regulating bacterial gene expression and virulence. *Infect. Immun.* **69**, 7197–7204.

Marinus, M. G. (1996). Methylation of DNA. In *Escherichia coli and Salmonella: Cellular and Molecular Biology* (F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger, eds.) 2nd edition, pp. 782–791. American Society for Microbiology, Washington, DC.

## Biography

Dr. Martin G. Marinus is a Professor in the Department of Biochemistry and Molecular Pharmacology at the University of Massachusetts Medical School in Worcester. His principal research interest is the molecular biological processes that involve DNA adenine methylation in *Escherichia coli*. He holds a Ph.D. from the University of Otago, New Zealand, and received his postdoctoral training at Yale University.

# DNA Mismatch Repair and Homologous Recombination

**Ivan Matic and Miroslav Radman**
*INSERM U571, Faculté de Médecine Necker-Enfants Malades, Université Paris V, Paris, France*

**Mismatches are mispaired or unpaired bases in the double-strand DNA molecule. Mismatches are generated as a consequence of errors during DNA replication or during homologous recombination involving nonidentical sequences. Specific mismatches can be formed by DNA damage. Enzymes that recognize and process mismatches have been identified in prokaryotes and eukaryotes. Different mismatch repair systems (MRS) are distinguished on the basis of their mismatch specificity and the size of excision tract that varies from single nucleotide to over a kilobase. The long-patch, also called the general, MRS (because it recognizes a variety of mismatches) is highly conserved during evolution. This repair system controls the fidelity of chromosomal replication by eliminating DNA biosynthetic errors and participates in the processing of some DNA lesions in transcription-coupled repair and in meiotic and mitotic recombination processes. The control of homologous recombination is particularly important because this fundamental biological process is essential for the maintenance of chromosomal integrity, generation of genetic diversity, proper segregation of meiotic chromosomes, and the speciation. Specific mismatches created by chemical modification of DNA bases (e.g., G:T mismatch due to deamination of 5-methyl-C to T or 8-oxo-G:A mismatch due to oxidation of G) are repaired by specialized short-patch MRS. When such mismatches occur in the course of recombination, their localized repair in heteroduplex DNA creates mosaic sequences, i.e., an apparent hyper-recombination effect.**

## Generalized Mismatch Repair Systems

### *Escherichia coli* Methyl-Directed Mismatch Repair Pathway

The best characterized generalized MRS is *Escherichia coli* methyl-directed MRS. It has been completely reconstituted *in vitro* and involves three dedicated proteins: MutS, MutL, and MutH. Other proteins that participate in the processing of mismatches are shared with other repair pathways. MutS protein recognizes and binds to mismatches, MutL associates with MutS-mismatch complex and activates MutH, which incises the newly synthesized strand at a nearby unmethylated 5′-GATC-3′ site. GATC sites are hemimethylated because methylation of adenine by Dam methylase lags behind replication by several minutes. Thus, MutH directs the mismatch repair process to the newly synthesized strand. Neither MutH nor nonmethylated GATC are required if a single strand break is present in the substrate DNA. The helicase II (MutU) unwinds the DNA allowing degradation of the displaced single-strand DNA, assuring the irreversibility of this repair process. The involvement of different exonucleases in this repair pathway depends on orientation of the nick relative to the mismatch. The repair process is finalized by DNA polymerase III and DNA ligase activity.

MutS protein recognizes seven of eight possible base pair mismatches, C-C mismatches (the least frequent replication error) being refractory. In addition, MutS protein binds up to four unpaired bases allowing for repair of frameshift errors. MutS does not recognize mismatches in a sequence-specific manner, although sequence context does influence the efficiency of repair. MutS protein binds to mismatches as a homodimer and has affinities for various mismatches that reflect the efficiency of *in vivo* repair. MutS proteins possess an intrinsic ATPase activity, which is essential for mismatch repair. The inactivation of *mutS*, or any other of *mut* genes coding for general MRS, show identical strong mutator phenotypes with $10^2$- to $10^3$-fold increased rates of transition (G:C → A:T and A:T → G:C) and frameshift mutations, as compared to mismatch repair proficient bacteria.

### Generalized Mismatch Repair in Eukaryotes

The knowledge about *E. coli* general MRS greatly facilitated identification and comprehension of homologous MR systems in other organisms. However, there are significant differences between these repair systems.

 © 2004, Elsevier Inc. All Rights Reserved. 

In most organisms, DNA strand recognition is not governed by DNA methylation. Dam methylase, coupled with the presence of *mutH* gene, has been identified in only a limited number of bacterial species. The mechanism of strand discrimination in other prokaryotes and eukaryotes is not yet elucidated.

Core functions of MRS, MutS, and MutL proteins are conserved in prokaryotes and eukaryotes. However, unlike its prokaryotic homologs, these proteins function as heteromers. The most extensively characterized eukaryotic generalized MRS is that of *Saccharomyces cerevisae*. In this organism, six MutS homologues (Msh1-6) have been identified. Msh1 functions in mitochondria, while others act in the nucleus. Heterodimers of the MutS homologs cooperate with heterodimers of MutL homologs to process different mismatches. Msh2–Msh6 (MutS$\alpha$) complex preferentially targets base pair mismatches and insertions/deletions of one or two nucleotides. Msh2–Msh3 (MutS$\beta$) binds 1–10 nucleotides large insertion/deletions loops.

Four MutL homologs (Mlh1-3 and Pms1) were identified in *S. cerevisae*. These proteins also form heterodimers. The majority of mismatch-repair activity is carried out by Mlh2-Pms1 heterodimer interacting with either MutS$\alpha$ or MutS$\beta$ complex. Mlh1 forms complexes with either Pms1, Mlh2, or Mlh3, each having distinct roles in the repair of mutational intermediates. As in prokaryotes, additional factors participate in *S. cerevisae* mismatch repair: proliferating cell nuclear antigen (PCNA; processivity factor for replication machinery) Exo1 ($5' \rightarrow 3'$ exonuclease), and the DNA polymerases $\delta$ and $\beta$.

In other eukaryotic organisms, invertebrates, vertebrates, and plants, multiple MutS and MutL homologs have also been identified. Different species contain different sets of homologs, without an obvious correlation to their position in the evolutionary tree. The eukaryotic mismatch-repair deficient mutants show mutator phenotypes similar to prokaryotic ones, i.e., increased rate of transition and frameshift mutations.

## Mismatch Repair System and Homologous Recombination

### Anti-Recombination Activity in *Escherichia coli*

Homologous recombination can be defined as interaction between two DNA sequences sharing extensive nucleotide sequence identity, present on a single or two different DNA molecules, that results in generation of mixed sequences derived from two parental ones. The homologous recombination process occurs in four basic steps: initiation, homologous pairing and DNA exchange, DNA heteroduplex extension, and resolution. The recombination process is initiated by combined action of a variety of nucleases and helicases that generate single-strand DNA. Single-strand DNA is substrate for proteins that catalyze homologous pairing and DNA exchange. The best studied homologous pairing and DNA exchange protein is *Escherichia coli* RecA.

RecA protein binds to single-strand DNA, or double-strand DNA containing single-strand DNA gaps, and forms a nucleoprotein complex containing one RecA protein monomer for every three nucleotides. RecA-single-strand DNA filaments engage search for homologous target DNA sequences. Scanning DNA for homologous sites is very fast and highly accurate even when they are very rare in the genome. *In vitro* data show that one helical turn of a RecA nucleoprotein filament containing approximately six RecA monomers and 15 bases of single-strand DNA is the functional unit sufficient to carry out the homology search. *In vivo* data show that RecA-catalyzed pairing is effective providing that the length of shared uninterrupted identity is at least 26 nucleotides.

After homologous pairing is achieved, the resultant joint molecules exchange homologous strands and establish heteroduplex DNA, where each parental DNA contributes one complementary strand. Heteroduplex DNA is extended by a unidirectional branch migration step. At this step of recombination, RecA protein tolerates DNA lesions, large heterologies, and up to 30% nucleotide sequence divergence between recombining molecules. However, pairing of nonidentical DNA sequences produces mismatched heteroduplex molecules, a substrate for MRS (Figure 1). *In vitro* experiments demonstrated that MutS and MutL proteins block RecA catalyzed strand-exchange and elongation of heteroduplex. *In vivo* MRS is capable of impeding recombination even between sequences with very low divergence. For example, transductional recombination between two serovars (Typhimurium and Typhi) of *S. enterica*, whose genomes differ only 1–2% at DNA sequence level, increases $10^2$- to $10^3$-fold in MRS deficient genetic background.

The antirecombination activity of the MRS is a consequence of blocking the RecA-catalyzed heteroduplex elongation but also of reducing the length of sequence identity needed for RecA-mediated homologous pairing and DNA exchange. The increased concentration of RecA protein does not prevent mismatch-repair antirecombination activity, whereas the overproduction of MutS and MutL proteins severely reduces recombination frequency between diverged DNAs (Figure 2).

Unlike the identical effect of MRS mutants on spontaneous mutagenesis, the inactivation of different MRS genes has distinct and characteristic effect on the recombination between diverged sequences. Very strong

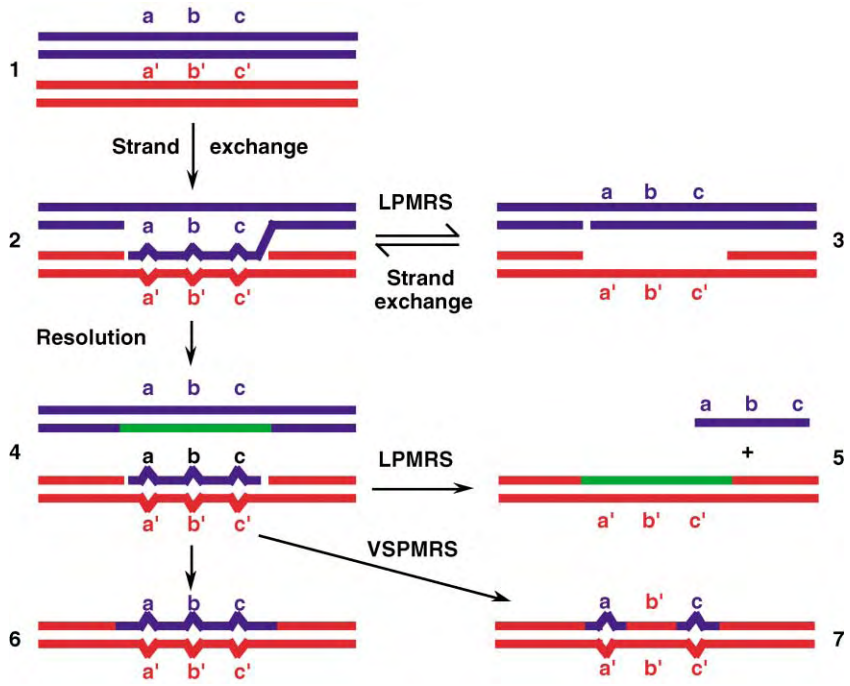



**FIGURE 1** Anti-recombination by long patch mismatch repair system (LPMRS) and hyper-recombination by very short patch mismatch repair system (VSPMRS). a/a′, b/b′, and c/c′ represent single nucleotide polymorphisms between two recombining DNA molecules (blue and red) (1). For simplicity, only one kind of strand exchange (asymmetric strand exchange) is shown; a:a′, b:b′ and c:c′ are mismatched bases in the heteroduplex intermediate (2). LPMRS can destroy mismatched heteroduplex before (3) of after (4 and 5) resolution of recombination intermediates. Heteroduplexes that escape LPMRS, and which are sealed by ligase (6), will be separated by subsequent replication producing one recombinant and one parental DNA molecule. If some of the mismatches (e.g., b:b′) in this heteroduplex molecule are substrate for VSPMRS, its activity will generate a complex recombinant on the repaired strand (7). Green lines represent tracts of DNA repair synthesis.

hyper-recombination effect is observed upon inactivation of *mutS* and *mutL* genes (Figure 2), while the effect of inactivation of *mutU* and *mutH* genes is relatively weak. The presence of single strand ends in recombination intermediates (Figure 1) can explain the weak effect of *mutH* gene inactivation. The helicase II can probably be replaced by other enzymes that are involved in recombination, e.g., RecG.

## Anti-Recombination Activity in *Saccharomyces cerevisae*

The anti-recombination activity of the eukaryotic MRS has been best characterized in *S. cerevisiae*, where it can inhibit recombination between diverged sequences both in mitosis and in meiosis. Even a single mismatch is sufficient for MRS to inhibit recombination, while additional mismatches have a cumulative negative effect on recombination efficiency. When DNA sequence divergence reaches several percent, the probability of a heteroduplex recombination intermediate to escape detection by the MRS is so low that the additional mismatches fail to increase anti-recombination activity. The MutS$\alpha$ or MutS$\beta$ complexes recognize the same types of mismatches in recombination intermediates as in replication intermediates. Although *msh2* and *mlh1* or *pms1* mutants have identical mutator phenotypes, *msh2* mutants exhibit higher recombination rates between diverged DNAs than do *pms1* or *mlh1* mutants. This indicates that Msh binding to mismatch may by itself inhibit the recombination process.

In *S. cerevisiae*, MRS processing of recombination intermediates results in gene conversion or complete destruction of recombination intermediates. Gene conversion is a genetic phenomenon in which information on one chromosome is replaced with information from the homologous chromosome, which remains unchanged (nonreciprocal recombination). In *S. cerevisae*, where it is possible to analyze all four products derived from a single meiosis, a non-Mendelian 3:1 segregation pattern of allelic sequences is diagnostic of a gene conversion event. In the absence of a functional MRS, mismatches in heteroduplex recombination intermediates are not repaired and the mismatch-containing DNA strands segregate at the first mitotic division following meiosis (postmeiotic segregation or PMS). The frequency of PMS reflects the repair efficiency for the particular mismatch. In *msh2*, *pms1*, *mlh1*, and to a lesser extent *msh3* and *msh6* mutants, meiotic gene

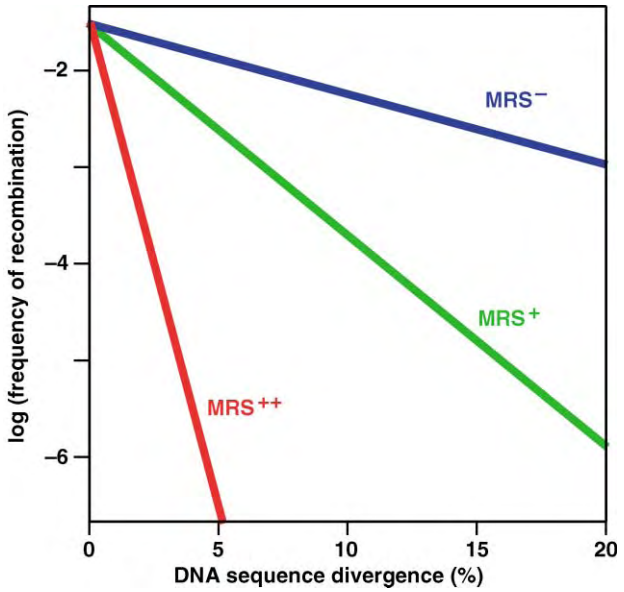



**FIGURE 2** The relationship between DNA sequence divergence, homologous recombination, and mismatch repair system (MRS). Conjugational crosses between enterobacterial strains and species show that linear increase of sequence divergence between recombining genomes results in the exponential decrease in recombination frequency. This relationship shows that an increase in DNA divergence increases the difficulty for RecA protein to find the sites for initiation of homologous pairing and strand exchange. The MRS activity reduces the apparent length of these sites and blocking the maturation of RecA-catalyzed recombination intermediates. Consequently, the inactivation of MRS decreases the slope of correlation between recombination and DNA divergence, i.e., increasing the frequency of recombination between diverged sequences. In contrast, the overproduction of the key MRS proteins (MutS and MutL) increases the slope ($MRS^{++}$), i.e., creates new genetic barriers.

conversion events are reduced while PMS events are elevated. Since the repair patch is shorter than the meiotic heteroduplex DNA, the repair process does not necessarily interfere with the formation of crossing overs. Although the same mismatch-repair machinery detects mismatches in both recombination and replication intermediates, some accessory proteins are probably unique to each type of repair. For example, PCNA appears to play a role in correcting DNA replication errors but has not been implicated in the correction of heteroduplex recombination intermediates.

MRS inhibits crossing-over between diverged chromosomes in interspecific hybrids during meiosis, and therefore it is also important for establishing and maintaining interspecific genetic barriers. In addition, the mismatch-triggered anti-recombination activity of eukaryotic MRS proteins inhibits ectopic interactions between diverged, repetitive DNA sequences during both mitosis and meiosis, and therefore it maintains genome stability by preventing genome rearrangements. Interestingly, mitotic recombination in yeast is more sensitive (in a mismatch repair-dependent manner) to low levels of sequence divergence than is meiotic recombination, which may help reinforce the strong bias for sister chromatid versus interhomolog interactions during mitosis.

Two *S. cerevisiae* MutS homologs Msh4 and Msh5 have completely lost the ability to participate in a standard mismatch repair reaction but have acquired novel, meiosis-specific roles. The loss of either MSH4 or MSH5 is associated with an approximately 50% reduction in meiotic crossing-over, in increased levels of homolog nondisjunction, and decreased viability of meiotic products. The Msh4 and Msh5 genes are in the same epistasis group and, like the other MutS homologs, physically interact to form heterodimers. As gene conversion levels are normal in *msh4* or *msh5* mutants, the Msh4-Msh5 complex is assumed to act late in recombination. Genetic data indicate that the Msh4–Msh5 heterodimer interacts specifically with the Mlh1-Mlh3 complex to promote meiotic crossing-over. Both *mlh1* and *mlh3* mutants exhibit a reduction in meiotic crossover events, and epistasis analysis indicates that Mlh1 and Msh4 act in the same crossover pathway.

## DNA Sequence Divergence, Mismatch Repair, Loss of Heterozygosity, and Cancer

Carcinomas evolve by accumulation of several somatic mutations, of which some are recessive (e.g., in tumor suppressor genes). Such mutations are expressed only when homozygous, i.e., when both alleles in diploid cells are inactivated by mutation. The transition from the heterozygous (m/+) to homozygous (m/m) or hemizygous (m/o) state usually occurs due to a chromosomal rearrangement leading to the loss of the functional gene copy (Figure 3). Such rearrangement can be due to (i) the loss of the (+) allele (e.g., a small or large deletion, or the loss of the entire chromosome), or (ii) a homologous recombination event, such as gene conversion of the allele to (m/m) or a mitotic crossover anywhere between the centromere and the allele (Figure 3). All these events, with the exception of gene conversion, lead to the loss of heterozygosity (LOH) initially present because of the maternal/paternal sequence divergence (about 0.1% of nucleotides in human population).

Two lines of experiments suggest that the functionally largely neutral sequence polymorphism between the two parental lines effectively suppresses the mitotic recombination mechanism for LOH:

(a) Gene targeting in mouse embryonic stem cells is highly sensitive to the natural sequence polymorphism of the parental strains. That suppression of mitotic

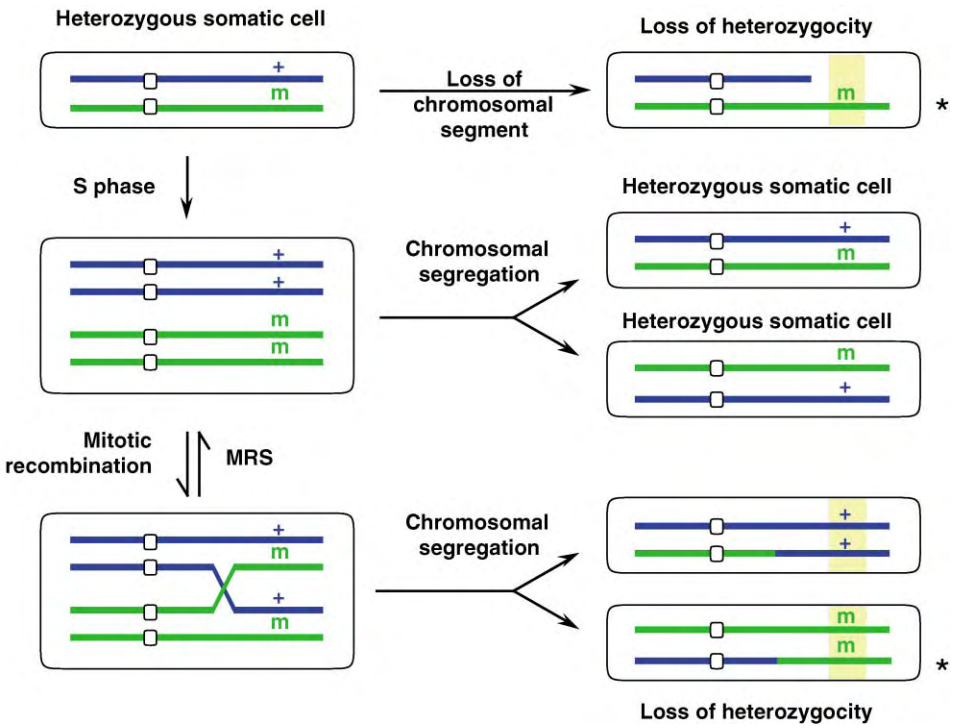



**FIGURE 3** DNA sequence divergence, mismatch repair, and loss of heterozygosity. Only one pair of homologous chromosomes is shown. The chromosome colors (blue and green) indicate the sequence polymorphism between the maternal and paternal line (0.1% in human population). The symbol (m) stands for a specific recessive mutation. Only cells (*) having lost the wild type (+) allele express the (m) mutation. Such cells show loss of heterozygocity (LOH; yellow). In human tumors, most LOH occurs by chromosomal rearrangements (top right). However, in inbred mice, with little or no sequence divergence between homologs, most LOH occurs by mitotic recombination. The recombination-suppressing effect of sequence polymorphism is relieved in MRS deficient mouse cells (see Figure 1). Because LOH is an obligate step in the expression of recessive mutations in tumor suppressor genes, sequence polymorphism protects against cancer due to MRS activity.

recombination can be relieved by the defect in mismatch repair, i.e., it appears that the mammalian mismatch repair acts to prevent mitotic recombination between nonidentical sequences.

(b) The expression of a recessive heterozygous (m/+) mutation in inbred, globally highly homozygous mice, occurs at elevated frequency *via* mitotic recombination, i.e., LOH from the crossover point to the end of the chromosome including the recessive mutation (Figure 3). In hybrids between different strains, the LOH frequency is much lower than in inbred strains. The decrease in LOH is accounted by the loss of mitotic recombination due to the activity of the MRS.

Thus, it appears that – like in bacteria and yeast – the editing (suppression) of homologous recombination by the MRS, based on the sequence divergence between the recombining sequences, functions also in mammalian cells. Such genetic barriers based on the genomic sequence polymorphism are expected to: (i) delay the process of carcinogenesis by delaying the expression of relevant recessive somatic and inherited mutations, and (ii) enhance sympatric speciation by creating genetic isolation on the germ line level.

## Evolutionary Role of the Control of Homologous Recombination by Mismatch Repair

Evolutionary conservation of key MRS functions illustrates the importance of this genetic system in the preservation of genomic integrity in prokaryotes and eukaryotes. In bacteria, the loss of MRS activity can provide transient selective advantage when adaptation is limited by the supply of mutations. However, the restoration of MRS proficiency is prerequisite for evolutionary success because upon adaptation, the load of deleterious mutations is no longer counterbalanced by the generation of beneficial mutations resulting in fitness reduction. In eukaryotes, the increased genomic instability resulting from MRS deficiency is apparently never advantageous. For example, in mammals, the loss of MRS activity is associated with male or female sterility and tumorigenesis.

Increased frequency of chromosomal rearrangements is a particularly deleterious phenotype of MRS deficiency in prokaryotes and eukaryotes. For example,

the inactivation of MRS increases 10-fold the frequency of chromosomal duplications resulting from recombination between *E. coli rhsA* and *rhsB* loci (0.9% divergent) and about $10^3$-fold gene conversion between *Salmonella enterica tufA* and *tufB* genes (about 1% divergent). The MRS-mediated recombination surveillance is particularly important for eukaryotes because their genomes contain a multitude of repetitive DNA sequences. MRS in *S. cerevisiae* prevents intra- and inter-chromosomal crossovers and gene conversions resulting from recombination between nonidentical DNA sequences.

By controlling homologous recombination, MRS controls also the process of speciation. In bacteria, MRS controls conjugational, transductional, and transformational recombination between strains and species, thus reducing gene flow between diverged populations. Similarly, by controlling crossing-over and chromosome segregation in meiosis, MRS prevents hybridization between diverged organisms resulting in reproductive isolation of closely related species.

## See Also the Following Articles

DNA Ligases: Mechanism and Functions • DNA Ligases: Structures • DNA Mismatch Repair and the DNA Damage Response • DNA Mismatch Repair Defects and Cancer • DNA Mismatch Repair: *E. coli* Vsr and Eukaryotic G–T Systems • DNA Mismatch Repair in Bacteria • DNA Mismatch Repair in Mammals • DNA Polymerase III, Bacterial • Mitosis • Recombination: Heteroduplex and Mismatch Repair *in vitro*

## Glossary

**homologous recombination** Interaction between two DNA sequences sharing extensive nucleotide sequence identity, present on a single or two different DNA molecules, that results in generation of mixed sequences derived from two parental ones. Such recombination events can be nonreciprocal (gene conversion or "patch" recombinants) or reciprocal (crossover or "splice" recombinants).

**meiosis** (from Greek, *meion*, smaller) Two successive nuclear and cellular divisions resulting in the reduction of the chromosome number from diploid to haploid. The products of meiosis are haploid gametes. Because chromosome segregation in meiosis requires physical attachment of homologous chromosomes (chiasmata), each chromosome in the gamete is recombinant.

**mismatch** Mispaired or unpaired bases in the double-strand DNA molecule.

**mitosis** (from Greek, *mitos*, thread) Nuclear and cellular division of a eukaryotic somatic cell resulting in the generation of two daughter cells having the same number of chromosomes as the parent cell.

**mutation** (from Latin *mutare*, to change) Any heritable modification of genetic material.

## Further Reading

Borts, R. H., Chambers, S. R., and Abdullah, M. F. (2000). The many faces of mismatch repair in meiosis. *Mutat. Res.* **451**, 129–150.

Chang, D. K., Metzgar, D., Wills, C., and Boland, C. R. (2001). Microsatellites in the eukaryotic DNA mismatch repair genes as modulators of evolutionary mutation rate. *Genome Res.* **11**(7), 1145–1146.

Datta, A., Hendrix, M., Lipsitch, M., and Jinks-Robertson, S. (1997). Dual roles for DNA sequence identity and the mismatch repair system in the regulation of mitotic crossing-over in yeast. *Proc. Natl Acad. Sci. USA* **94**, 9757–9762.

de Wind, N., Dekker, M., Berns, A., Radman, M., and te Riele, H. (1995). Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* **82**, 321–330.

Friedberg, E. C., Walker, G. C., and Siede, W. (1995). *DNA Repair and Mutagenesis*. ASM Press, Washington, D.C.

Harfe, B. D., and Jinks-Robertson, S. (2000). Mismatch repair proteins and mitotic genome stability. *Mutat. Res.* **451**, 151–167.

Hunter, N., Chambers, S. R., Louis, E. J., and Borts, R. H. (1996). The mismatch repair system contributes to meiotic sterility in an interspecific yeast hybrid. *EMBO J.* **15**, 1726–1733.

Jones, M., Wagner, R., and Radman, M. (1987). Mismatch repair and recombination in *E. coli*. *Cell* **50**, 621–626.

Matic, I., Taddei, F., and Radman, M. (1996). Genetic barriers among bacteria. *Trends Microbiol.* **4**, 69–73.

Modrich, P., and Lahue, R. (1996). Mismatch repair in replication fidelity, genetic recombination and cancer biology. *Annu. Rev. Biochem.* **65**, 101–133.

Rayssiguier, C., Thaler, D. S., and Radman, M. (1989). The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. *Nature* **342**, 396–401.

Shao, C., Yin, M., Deng, L., Liskay, R. M., Stambrook, P. J., Doetschman, T. C., and Tischfield, J. A. (2002). Loss of heterozygosity and point mutation at Aprt locus in T cells and fibroblasts of Pms2-/- mice. *Oncogene* **21**, 2840–2845.

Vulic, M., Dionisio, F., Taddei, F., and Radman, M. (1997). Molecular keys to speciation: DNA polymorphism and the control of genetic exchange in enterobacteria. *Proc. Natl Acad. Sci. USA*. **94**, 9763–9767.

## Biography

Miroslav Radman is Professor of cell biology at the Medical School Necker of the University Paris 5. He is also the Director of the research Unit No. 571 of INSERM entitled "Evolutionary and Medical Molecular Genetics." He is a member of the EMBO, Academia Europea, French Academy of Science and Croatian Academy of Sciences and Arts. He is a pioneer in the study of DNA repair and mutagenesis, in particular of mutagenic SOS repair and the antimutagenic mismatch repair.

Ivan Matic is an Associate Research Professor of the French National Center for Scientific Research (CNRS). He directs the research group "Evolution of Enterobacteria" in the INSERM Unit No. 571 at the Medical School Necker, University Paris 5. He studies the mechanisms of mutation and recombination in bacteria, as well as their role in adaptive evolution of bacteria.

# DNA Mismatch Repair and the DNA Damage Response

**Guo-Min Li and Steven R. Presnell**
*University of Kentucky Medical Center, Lexington, Kentucky, USA*

**DNA mismatches are base-pairing errors that deviate from the Watson-Crick rules that stipulate that A pairs with T and G with C. In addition, small insertion/deletion structures, in which one DNA strand contains a small number of unpaired nucleotides, are also considered mismatches. Mismatched base pairs occur as errors during the normal course of DNA replication and recombination, and they are mutagenic if left uncorrected. DNA mismatch repair (MMR) is a cellular process that rectifies mismatches to yield correct Watson-Crick base pairs. MMR is an important genome maintenance system because defects in the system cause genomewide instability and have been implicated in the development of certain types of human cancer. In the past, the ability of the MMR system to correct DNA heteroduplexes has been considered the primary mechanism by which it contributes to genomic stability. However, more recent studies indicate that the MMR system also contributes to genomic stability by mediating protective cellular responses to DNA damage.**

## Mismatch Repair Proteins and Their Role in Mismatch Correction

The eukaryotic MMR pathway is similar to the *Escherichia coli* MutHLS pathway with respect to features of mechanism and involvement of several homologous activities. In fact, many of the eukaryotic MMR components were initially identified by their sequence homology to the *E. coli* MMR proteins (see Table I). The eukaryotic MMR proteins are known to include the MutS homologues (MutS$\alpha$, MutS$\beta$), the MutL homologues (MutL$\alpha$, MutL$\beta$, MutL$\gamma$), exonuclease I (ExoI), replication protein A (RPA, a eukaryotic, SSB (single strand DNA-binding protein)), and DNA polymerase $\delta$. Although eukaryotic homologues of the bacterial MutH and helicase II have not yet been identified, studies in human nuclear extracts have demonstrated that the human MMR reaction possesses a mechanism similar to that of *E. coli*; that is, the repair is targeted to the newly synthesized strand and can occur bidirectionally. The eukaryotic system appears, however, to be more complex than the *E. coli* pathway. For example, whereas only a single form of MutS or MutL in *E. coli* has been documented, at least two MutS homologues and three MutL homologues have been identified in eukaryotic cells, each of which is a heterodimer. For example, MutS$\alpha$ consists of the polypeptides MSH2 and MSH6, and MutL$\alpha$ comprises MLH1 and PMS2 (see Table I). These MutS and MutL heterodimers, although redundant, play special roles in MMR subpathways. In human cells, MutS$\alpha$ and MutL$\alpha$ are the most abundant species among the MutS and MutL heterodimers, respectively.

## Mismatch Repair Function is Required for Drug Cytotoxicity

Although MMR is well known for its role in correcting biosynthetic errors, other important roles for MMR proteins are being recognized; one of these is the mediation of programmed cell death (or apoptosis) in cells with heavily damaged DNA. This apoptotic function of the MMR system was realized from both basic and clinical studies on how chemical or physical DNA damaging agents induce tumor cells to undergo cell death.

Treatment of cells with chemical DNA-damaging agents, such as the alkylating agents, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), temozolomide, or procarbazine, leads to increased amounts of cell death. For this reason, these cytotoxic agents are often used in chemotherapy to destroy rapidly growing tumor cells. There is great interest in the cancer research community in understanding the mechanism by which these agents cause cell death. Interestingly, it has been found almost universally that, whereas cells that are proficient in MMR are sensitive to these agents, cells that are deficient in MMR are more resistant to killing by these agents. This was first observed with *E. coli* in the 1980s when *E. coli* MMR mutants (*mutS*$^-$ or *mutL*$^-$) were found to be more resistant to killing by

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**MMR Components and Their Function**[a]

| *E. coli* | Human | Function |
|---|---|---|
| MutS | MutS$\alpha$ (MSH2-MSH6)[b]<br>MutS$\beta$ (MSH2-MSH3) | DNA mismatch/damage sensor |
| MutL | MutL$\alpha$ (MLH1-PMS2)[b]<br>MutL$\beta$ (MLH1-PMS1)<br>MutL$\gamma$ (MLH1-MLH3) | Molecular chaperon and/or DNA damage signal transducer? |
| MutH | ? | Strand discriminator |
| Helicase II | ? | Unwinding DNA helix |
| ExoI, ExoVII, ExoX, RecJ | ExoI, ? | Removing mispaired base |
| Pol III holoenzyme | Pol $\delta$, PCNA | Repair DNA synthesis |
| SSB | RPA | Protecting template DNA from degradation |
| DNA ligase | ? | Nick ligation |

[a] ExoI, exonuclease I; PCNA, proliferating cell nuclear antigen; Pol $\delta$, polymerase $\delta$; RPA, replication protein A; ?, not yet identified.
[b] Major components in cells.

MNNG than were wild-type bacterium. In addition, resistance to cytoxic agents such as cisplatin (a cross-linking agent) and MNNG has also been observed in human cells that are defective in MutS$\alpha$ or MutL$\alpha$. An interesting example is afforded by the development of the lymphoblastoid cell line MT1. This cell line was derived from the TK6 cell line by selection with a high dose of MNNG. MT1 cells are 500-fold more resistant to killing by this agent than the parental cells and exhibit an elevated spontaneous mutation rate. The MT1 cell line was subsequently found to be defective in *MSH6*, the gene that encodes a subunit of MutS$\alpha$ that is necessary for MMR. Conversely, many human tumor cells that are defective in MMR have been found to be relatively resistant to alkylating agents. For example, the *MLH1*-defective colorectal tumor cell line HCT116 is resistant to killing by MNNG, and the MNNG resistance is lost by these cells when they have received a wild-type copy of the *MLH1* gene by chromosome transfer. Therefore, the cytotoxicity of DNA damaging agents is dependent on a functional MMR system.

## Mismatch Repair Proteins Promote DNA Damage-Induced Cell Cycle Arrest and Apoptosis

Normal cells are known to undergo growth arrest at cell-cycle checkpoints when exposed to DNA-damaging agents. This response allows cells to respond to DNA damage either by repairing the damage or by committing programmed cell death (apoptosis). Interestingly, cells defective in MMR fail to arrest at crucial checkpoints after exposure to DNA-damaging agents. For example, MMR-proficient cells undergo growth arrest at the G2 phase of the cell cycle after treatment with alkylating agents, but MMR-deficient cells do not. The G2 phase arrest in MMR-proficient cells has been found to be associated with apoptosis. As illustrated in Figure 1, when wild-type TK6 cells and MutS$\alpha$-deficient MT1 cells were treated with MNNG, apoptotic cell death was observed in TK6 cells (see the pattern of DNA

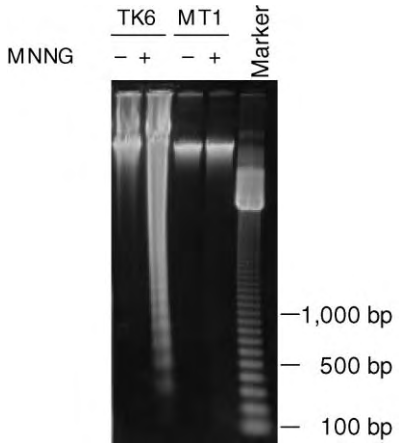



**FIGURE 1** MNNG induces apoptosis in MMR-proficient cells. MMR-proficient TK6 and MMR-deficient MT1 cells were treated with MNNG for 1 h and then cultured in fresh medium for 24 h before harvesting. Genomic DNAs isolated from MNNG-treated (+) and untreated cells (−) were electrophoresed in a 2% agarose gel and detected by UV-illumination in the presence of ethidium bromide. Notice that DNA fragmentation is observed in MNNG-treated TK6 cells, but not in MNNG-treated MT1 cells. This DNA fragmentation pattern is diagnostic of apoptosis, or programmed cell death.

fragmentation, a classical characteristic of apoptosis), but not in MT1 cells. Similarly, MutL$\alpha$ is also required for alkylating agent-induced apoptosis. This MMR-dependent apoptotic response can be also induced by other DNA-damaging agents, including cisplatin, certain environmental carcinogens, and ionizing radiation. In addition to cell lines, DNA-damage-induced MMR-dependent apoptosis also occurs in classic laboratory animals such as the mouse and the soil nematode *Caenorhabditis elegans*.

How is the MMR pathway involved in the apoptotic response? Although the details are far from clear, MMR initiation factors may play a crucial role in this response by recognizing DNA damage. For example, it has been established that the *E. coli* mismatch recognition protein MutS and its eukaryotic homologues recognize DNA bases damaged or modified by a variety of agents. These agents include MNNG, cisplatin, chemical carcinogens, oxidative free radicals, and ultraviolet (UV) light. These damaged DNA bases, or DNA adducts, possess structures different from regular bases, thereby causing conformational changes in the DNA duplex. For example, whereas the 8-hydroxyguanine adduct generated by ionizing radiation is approximately the same size as guanine, the guanine adduct of benzo[a]pyrene dihydrodiol epoxide, an environmental chemical carcinogen, is twice as large as guanine. However, a common feature shared by these DNA adducts, as well as DNA base–base mismatches, is that they more or less distort the structure of the DNA helix. This distortion may constitute the basis of their recognition by MutS and its eukaryotic homologues.

## Mismatch Repair Proteins Interact with Apoptotic Transducer in Response to DNA Damage

Apoptosis is normally mediated by the transcription factor p53 and its cousin, p73. Recent studies have indicated that both proteins are also implicated in MMR-dependent apoptosis. Upon treating cells with DNA damaging agents (e.g., MNNG), increased expression of p53 has been noted in MMR proficient cells, but not in cells defective in either MutS$\alpha$ or MutL$\alpha$. The increased level of p53 is due to its phosphorylation by a protein kinase, whose activation is dependent on a functional MMR system. In addition to p53, cells also use p73 for MMR-dependent apoptotic response. Recent studies have shown that phosphorylation and stabilization of p73 occur in an MMR-dependent manner during cellular response to cisplatin-induced DNA damage. The phosphorylation of p73 is carried out by the c-Abl protein kinase and the stabilization of p73 requires the interaction of the protein with the MutL homologue protein, PMS2. These observations indicate that MMR-dependent apoptosis in response to DNA damage involves a signaling cascade.

## Mismatch Repair-Mediated Apoptosis Eliminates Damaged Cells from Tumorigenesis

The molecular events involved in the MMR-dependent apoptotic response have not yet been established. However, based on what is already known, the apoptotic signaling is probably initiated by binding of MMR proteins to DNA adducts. This event stimulates the interactions between MMR proteins and an apoptotic transducer, p53 or p73. Two possible mechanisms for this signaling process are depicted in Figure 2. One model proposes that repetitive attempts by MMR to remove a DNA adduct in the template DNA strand cause cell death. DNA adducts in the template strand can pair with appropriate bases or lead to mispairs during DNA replication. MutS$\alpha$, along with MutL$\alpha$, recognizes these unusual base pairs as mismatches and provokes a strand-specific MMR reaction. However, because MMR is always targeted to the newly synthesized strand, adducts in the template strand cannot be removed and thus unusual base pairs reform upon DNA resynthesis during repair. As a result, the repair cycle can be perpetually reinitiated. Such a futile repair cycle may signal cells to switch on apoptotic machinery. Alternatively, the death signal could come from the binding of MutS$\alpha$/MutL$\alpha$ to DNA adducts in the replication fork, the unwound DNA helix, or both and may be unrelated to the repair process. These protein–DNA adduct complexes may block DNA transactions such as replication, transcription, and repair and could be recognized as a signal for cell-cycle arrest and death. In both models, the action on DNA adducts by MMR proteins activates protein kinases to phosphorylate apoptotic transducers such as p73 and p53 that have just established their physical interactions with MMR proteins upon the MMR processing of DNA adducts. The phosphorylation of p73/p53 activates apoptotic machinery and takes the cell down the road to eventual death.

The MMR pathway is well known for its function of promoting genomic stability. The newly identified apoptotic function of MMR, however, may be as important as its replication-fidelity function for maintaining genomic stability. Normally, base excision repair and nucleotide excision repair pathways are responsible for the repair of DNA damage induced by

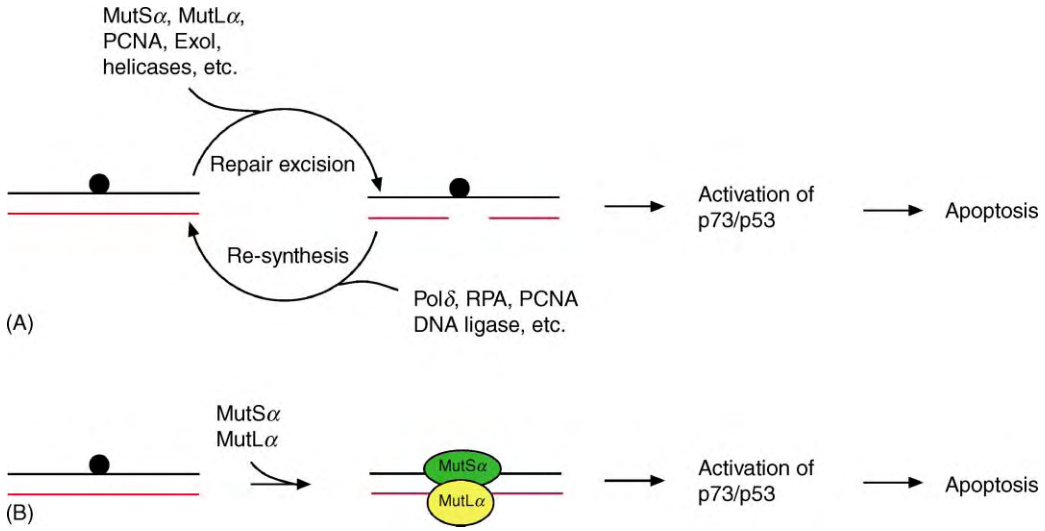



**FIGURE 2** Proposed models of how MMR mediates apoptosis in response to DNA damage. (A) The futile repair cycle model. The adducted base (solid circle) could pair with an appropriate base or lead to a mispair during DNA replication. This abnormal structure can be recognized by MutSα (MSH2–MSH6) and provoke a strand-specific MMR reaction. However, because MMR can be only targeted to the newly synthesized strand (red line), the offending adduct in the template strand (black line) cannot be removed and will initiate a new cycle of MMR upon repair resynthesis. Such a futile repair cycle stimulates an interaction between MMR proteins and apoptotic transducers, including ATM/ATR, c-Abl, and p73/p53. These interactions may activate the apoptotic machinery. (B) The blockage model. The binding of MutSα/MutLα to a DNA adduct in the replication fork, the unwound DNA helix, or both could block DNA transactions such as replication, transcription, and repair. The blockage promotes MMR proteins to interact with apoptotic transducers to switch on apoptotic machinery. ExoI, exonuclease I; PCNA, proliferating cell nuclear antigen; Pol δ, polymerase δ; RPA, replication protein A.

physical and chemical agents. However, when excision repair pathways are not available or there is too much damage to be repaired, genomic DNA is in danger of accumulating a large number of mutations, a process that is tumorigenic. Eliminating these damaged cells from the body would be beneficial. It is the MMR system that initiates the elimination of these pre-tumorigenic cells by promoting apoptosis. The inability of the MMR system to commit genetically damaged cells to apoptosis may contribute to a molecular basis for cancer development.

## See Also the Following Articles

Cell Death by Apoptosis and Necrosis • DNA Mismatch Repair Defects and Cancer • DNA Mismatch Repair: *E. coli* Vsr and Eukaryotic G–T Systems

## Glossary

**apoptosis** An active cell death process that requires RNA and protein synthesis to specifically digest cellular DNA into nucleosomal fragments; also known as programmed cell death.

**cytotoxicity** The degree to which an agent possesses a specific destructive action on certain cells or the possession of such action; often used when referring to the action of antineoplastic drugs that selectively kill dividing cells.

**DNA mismatch** Non-G:C or -A:T DNA base pairs or small insertion/deletion (unpaired) nucleotides in duplex DNA; also called a heteroduplex.

**DNA mismatch repair** A DNA repair pathway that specifically coverts mismatched DNA bases (heteroduplexes) into normal G:C or A:T base pairs (homoduplexes).

**tumorigenesis** The process that generates tumors.

## Further Reading

Aquilina, G., and Bignami, M. (2001). Mismatch repair in correction of replication errors and processing of DNA damage. *J. Cell Physiol.* **187**, 145–154.

Bellacosa, A. (2001). Functional interactions and signaling properties of mammalian DNA mismatch repair proteins. *Cell Death Differ.* **8**, 1076–1092.

Karran, P., and Bignami, M. (1994). DNA damage tolerance, mismatch repair and genome instability. *Bioessays* **16**, 833–839.

Li, G. M. (1999). The role of mismatch repair in DNA damage-induced apoptosis. *Oncol. Res.* **11**, 393–400.

Modrich, P. (1997). Strand-specific mismatch repair in mammalian cells. *J. Biol. Chem.* **272**, 24727–24730.

## Biography

Guo-Min Li is an Associate Professor in the Department of Pathology at the University of Kentucky. His principal research interests are in mammalian DNA mismatch repair and its role in DNA damage response. He holds a Ph.D. from Wayne State University and received his postdoctoral training at Duke University His laboratory was among the first to demonstrate that the recognition and the processing of DNA adducts by mismatch repair proteins trigger apoptosis.

Steven R. Presnell is a Research Associate in Dr. Li's laboratory. He holds a Ph.D. from the Georgia Institute of Technology and received his postdoctoral training at the University of North Carolina, Chapel Hill.

# DNA Mismatch Repair Defects and Cancer

**Richard D. Kolodner**
*Ludwig Institute for Cancer Research, University of California, San Diego School of Medicine, La Jolla, California, USA*

**A major source of mutagenic mispaired bases is misincorporation errors that occur during DNA replication. If such errors are not repaired prior to the next round of DNA replication, they result in mutations. These misincorporation errors are normally corrected by DNA mismatch repair (MMR). MMR recognizes the resulting mispaired base in DNA and excises the misincorporated base from the newly synthesized DNA strand, which is then resynthesized. This process significantly increases the fidelity of DNA replication. Because of the critical role of MMR in suppressing DNA replication errors, MMR defects cause an increased rate of accumulating mutations. Such mutations can activate oncogenes or inactivate tumor suppressor genes and contribute to the process of carcinogenesis.**

## Introduction

It has been demonstrated that increased mutation rates resulting from DNA replication errors (Figure 1) can play a role in the development of cancer by the discovery that (1) hereditary nonpolyposis colorectal carcinoma (HNPCC) can be caused by inherited mutations in some genes encoding mismatch repair (MMR) proteins, (2) somatically acquired MMR defects underlie some sporadic cancers, and (3) mice containing mutations in MMR genes or mutations that decrease the fidelity of the replicative DNA polymerases result in increased cancer susceptibility. This entry briefly reviews our understanding of MMR.

## Proteins Involved in Mismatch Repair

### MutS Homologue Proteins Involved in Mismatch Repair

A model has been proposed for eukaryotic MMR in which mispaired bases in DNA are recognized by heterodimeric complexes of proteins that are homologues of the bacterial MutS protein (Figure 2). MutS is the bacterial MMR protein that recognizes mispaired bases in DNA (Mut is an abbreviation for Mutator, because mutations in the *mutS* gene cause high mutation rates or a mutator phenotype). Three different MutS homologues, called MSH proteins, function in MMR. These three different MSH proteins (MSH2, MSH3, and MSH6) form two different heterodimeric complexes, the MSH2–MSH6 (MutSalpha) and MSH2–MSH3 (MutSbeta) complexes (see Figure 2). Extensive genetic studies indicate that the MSH2–MSH6 (MutSalpha) complex is the major mismatch recognition complex that functions in repair of base:base mispairs and a broad spectrum of insertion/deletion mispairs. The MSH2–MSH3 (MutSbeta) complex appears to be able to substitute for the MSH2–MSH6 (MutSalpha) complex in the repair of many insertion/deletion mispairs and may be primarily responsible for the repair of larger insertion/deletion mispairs.

The MSH2–MSH6 (MutSalpha) and MSH2–MSH3 (MutSbeta) complexes have been purified and extensively studied. Consistent with genetic studies, MSH2–MSH6 (MutSalpha) is able to support the repair of both base:base mispairs and insertion/deletion mispairs in *in vitro* MMR reactions, whereas MSH2–MSH3 (MutSbeta) is only able to support the repair of insertion/deletion mispairs. The MSH2–MSH6 (MutSalpha) and MSH2–MSH3 (MutSbeta) complexes have also been shown to preferentially bind to mispaired bases in DNA. Upon mispair recognition they appear to form a ring around the DNA that can then move along the DNA and function in MMR. The MSH2–MSH6 (MutSalpha) complex binds to both base:base mispairs and insertion/deletion mispairs, whereas the MSH2–MSH3 (MutSbeta) complex appears to bind only insertion/deletion mispairs. There is some indication that the *Saccharomyces cerevisiae* and mouse MSH2–MSH3 (MutSbeta) complex may have a relatively greater ability to function in the repair of single base insertion/deletion mispairs than the human MSH2–MSH3 (MutSbeta) complex.

 © 2004, Elsevier Inc. All Rights Reserved. 

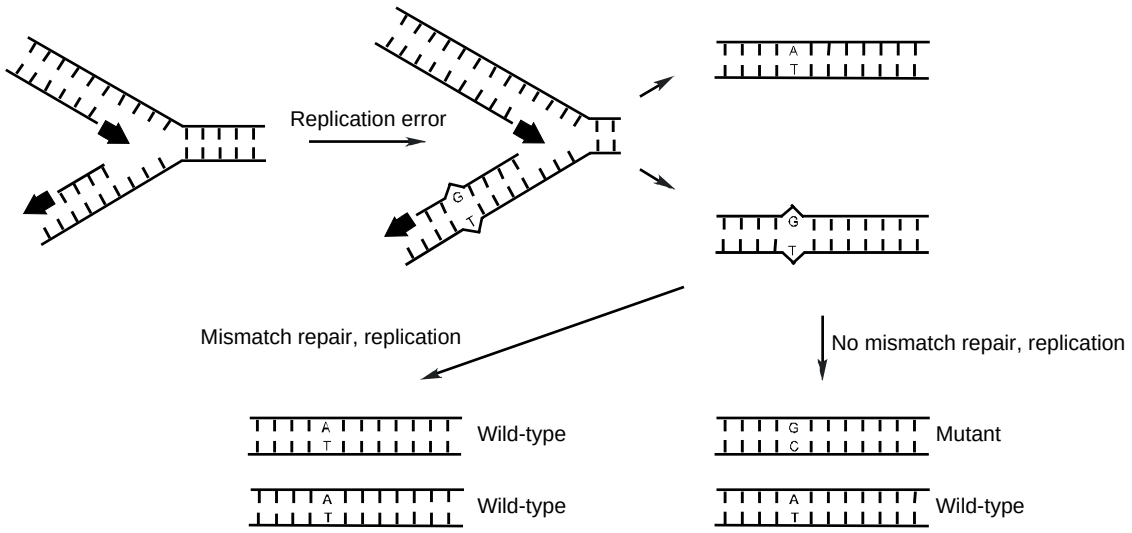



**FIGURE 1** The origin of mutations as a result of errors during DNA replication. A DNA molecule in the process of being copied by DNA polymerases (large arrows) is illustrated at the upper left. Such structures are often called replication forks. After an error occurs in which a G is misincorporated opposite a T, as illustrated at the upper center, replication completes and yields one correctly paired DNA and one mispaired DNA as illustrated at the upper right. If the mispaired DNA is then replicated before it is repaired, a mutant and a wild-type DNA result as illustrated at the lower right. If mismatch repair occurs prior to DNA replication, then two wild-type DNAs result as illustrated at the lower left.

One of the key properties of the MSH protein complexes [MSH2–MSH6 (MutSalpha) and MSH2–MSH3 (MutSbeta)] is that they are partially redundant. This is relevant to the observation that defects in MMR can cause cancer (discussed later). Because MSH2 is the only protein present in both complexes, defects in the MSH2 gene completely inactivate MMR (see Figure 2). As a consequence, MSH2 defects result in increased rates of accumulation of both base substitution and frameshift mutations. Interestingly, short mononucleotide repeat sequences are often found within genes, and frameshift errors that occur by the copying of such sequences during replication are the most frequent misincorporation errors made by DNA polymerases. Thus, in MSH2-defective cells, frameshift mutations in mononucleotide repeats often occur more frequently than base substitution mutations. MSH6 and MSH3 gene defects inactivate only the MSH2–MSH6

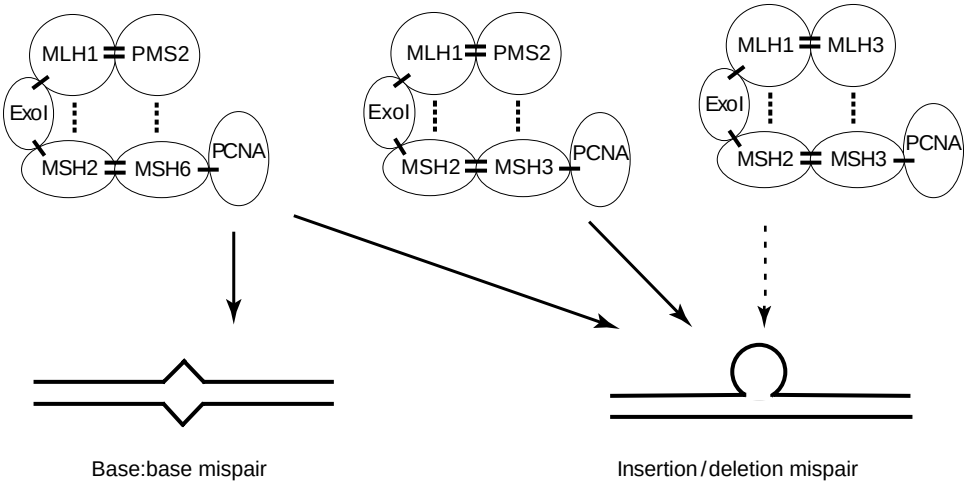



**FIGURE 2** Illustration of the protein complexes that function in mismatch repair. The circles and ovals represent the MSH2, MSH3, MSH6, MLH1, MLH3, PMS2, exonuclease I, and PCNA proteins, as indicated. The double solid connecting lines indicate known stable protein–protein interactions, the single solid connecting lines indicate known, less stable protein–protein interactions, and the dashed connecting lines indicate interactions between protein assemblies in which the exact protein contacts are not yet known. Below these illustrated protein complexes are the mispaired based containing DNAs they interact with during mismatch repair. The solid connecting arrows indicate the major repair reactions, and the dashed connecting arrow indicates a minor repair pathway.

(MutSalpha) and MSH2–MSH3 (MutSbeta) complexes, respectively. Because of the partially overlapping mispair recognition specificity of each complex, defects in MSH6 cause only large increases in the rate of base substitution mutations, like that seen in MSH2 mutants, and cause small increases in the rate of frameshift mutations relative to that seen in MSH2 mutants. Defects in MSH3 have very little effect on mutation rates except for frameshift mutations in repeating sequences where the repeat unit length is 2 bases and larger. Dinucleotide and larger repeats are found less frequently in sequences encoding genes than short mononucleotide repeats, indicating that MSH3 defects should have little effect on the accumulation of mutations within the majority of genes.

## MutL Homologue Proteins Involved in Mismatch Repair

MutL is a bacterial protein that is required for MMR; it is known to interact with MutS and then activate other proteins that function in MMR. There are three MutL homologues, called MLH proteins: MLH1, PMS2 (called PMS1 in *S. cerevisiae*), and MLH3, for which there is good evidence for a function in MMR in eukaryotes. These proteins form two different heterodimeric complexes, MLH1–PMS2 (MutLalpha) and MLH1–MLH3 (see Figure 2). Biochemical experiments have shown that the MLH1–PMS2 (MutLalpha) complex forms a higher order complex with both MSH2–MSH6 (MutSalpha) and MSH2–MSH3 (MutSbeta) when these MSH complexes interact with DNA. Presumably the MLH complex then acts to activate other components of MMR; however, little is yet known about this process. Neither is much yet known about the biochemical properties of the MLH1–MLH3 complex. A fourth MutL-related protein, human PMS1 (called MLH2 in *S. cerevisiae*), which appears to be able to form a complex with MLH1, was initially suggested to function in MMR. Genetic and biochemical studies performed to date, however, have failed to provide evidence that this protein plays an important role in MMR.

Genetic studies have shown that the MLH1–PMS2 (MutLalpha) complex is the major MutL-related complex that functions in MMR. The MLH1–MLH3 complex appears to play only a minor role in MMR. Like the situation with the two different MSH complexes discussed previously, the observation that two different MutL-related protein complexes function in MMR indicates that defects in each of the three MutL-related genes may cause different MMR defects. Because MLH1 is the only protein present in both MutL-related complexes, only defects in the MLH1 gene completely inactivate MMR (see Figure 2). However, since the MLH1–PMS2 (MutLalpha) complex is the major complex that functions in MMR, defects in the PMS2 gene cause almost as strong a defect in MMR as do defects in the MLH1 gene. In contrast, because the MLH1–MLH3 complex plays a minor role in MMR, defects in the MLH3 gene cause only a partial defect in MMR, resulting in a small but significant increase in the rate of accumulating frameshift mutations in mutant cells. Defects in human PMS1 or its *S. cerevisiae* homologue MLH2 cause little if any significant increase in mutation rate.

## Other Proteins Implicated in Mismatch Repair

One of the important problems in the study of eukaryotic MMR is the identification of the other proteins required for MMR. Some progress has been made in this area, but unfortunately, little is known about how MMR initiates and how the newly synthesized DNA strand is recognized, nor has eukaryotic MMR been reconstituted from purified proteins.

DNA polymerase delta is required for the DNA synthesis step of MMR; similarly, there is also a requirement for its accessory factors proliferating cell nuclear antigen (PCNA), replication protein A (RPA/RFA), and replication factor C (RFC). PCNA has also been suggested to be involved in MMR at an early step prior to the resynthesis step. PCNA interacts with the MSH2–MSH6 (MutSalpha) and MSH2–MSH3 (MutSbeta) complexes through a specific motif located in the MSH6 and MSH3 subunits. This has led to the suggestions that PCNA may be required for the activity of the MSH2–MSH6 (MutSalpha) and MSH2–MSH3 (MutSbeta) complexes or that PCNA may target these MMR proteins to regions of newly replicated DNA. In considering the possibility that defects in genes encoding DNA polymerase delta, PCNA, RPA, and RFC might play a role in cancer susceptibility (see following discussion), it is important to note that each of these genes is an essential gene that would preclude mutations that cause complete loss-of-function defects.

In bacteria, at least four exonucleases have been shown to be able to function in the degradation step of MMR; each can substitute for all of the others. This redundancy of exonucleases appears to occur in eukaryotic MMR. Exonuclease 1 is a 5′ to 3′ double-strand DNA-specific exonuclease encoded by the EXO1 gene that is thought to be one of a number of redundant exonucleases that function in eukaryotic MMR. Exonuclease 1 physically interacts with both MSH2 and MLH1, and genetic studies have suggested that exonuclease 1 may in some way help to assemble higher order protein complexes that function in MMR. Biochemical studies have shown that exonuclease 1

can function in the excision step of MMR *in vitro* and plays a role in excision in both 5′ to 3′and 3′ to 5′ directions. Consistent with the view that exonuclease 1 is only one of many exonucleases that function in MMR, mutations in the EXO1 gene cause only partial MMR defects. Other exonucleases that have been suggested to function in MMR are the endo/exonuclease FEN1/RAD27 and the 3′ to 5′ editing exonuclease functions of DNA polymerases delta and epsilon. However, whether these latter three exonucleases play a direct role in MMR has been difficult to determine because these exonucleases all function in other critical aspects of DNA synthesis.

## Defects in Mismatch Repair Cause Increased Cancer Susceptibility

HNPCC is a common inherited cancer susceptibility syndrome that in its most striking examples is characterized by a dominant mode of transmission, high penetrance, significantly earlier age of onset than sporadic cancers, multiple primary tumors, and a high proportion of colorectal as well as many other types of cancer, including endometrial, genitourinary, extracolonic GI cancers, ovarian, brain, and sebaceous skin tumors. A number of clinical criteria for the identification of HNPCC have been used, ranging from the highly stringent Amsterdam criteria (see Table I) that identify kindreds based on clustering of colorectal cancer and an early age of onset, to less restrictive criteria that allow inclusion of individuals with a less well documented family history of cancer and families with colorectal cancer and HNPCC-associated cancers as well as early onset colorectal cancer cases without a documented family history of cancer. DNA from HNPCC tumors often shows a high frequency of frameshift mutations in microsatellite sequences, a phenotype called microsatellite instability (MSI). MSI is an important diagnostic tool for identifying cases of HNPCC that are due to MMR defects. Tumors showing a high proportion of unstable microsatellite sequences are often designated MSI-High (MSI-H).

A high proportion of HNPCC cases defined by the Amsterdam criteria show tumor MSI, whereas a lower proportion of HNPCC cases defined by other clinical criteria show tumor MSI. Recent genetic studies suggest that almost all HNPCC cases showing tumor MSI can be accounted for by inherited defects in the MSH2 gene or the MLH1 gene. These two genes encode the common subunits of the MutS-related and MutL-related family of heterodimeric complexes, respectively, that function in MMR. The types of inherited mutations observed in these two genes include frameshift and nonsense mutations (protein-truncating mutations), missense mutations (protein sequence changes), and deletion mutations; deletion mutations appear to be more prevalent in MSH2 than MLH1, and missense mutations appear to be more prevalent in MLH1 than MSH2. There are no simple, routine molecular diagnostic methods for diagnosing mutations that cause HNPCC. However, in combination with an appropriate family history of cancer, complete MSH2 and MLH1 gene sequencing and deletion analysis along with testing of HNPCC tumors for MSI using a standard set of microsatellite markers and analysis of tumors for loss of MSH2 or MLH1 protein expression using immunohistochemistry (sometimes called IHC analysis) have proven to be useful in detecting MMR defects in suspected HNPCC families. Through the application of these methods, most HNPCC cases that have MSI-H tumors have been linked to a mutation in MSH2 or MLH1. In addition, in some cases in which inherited mutations have not been found, altered expression of MSH2 or MLH1 has been identified, indicating the presence of some type of MSH2 or MLH1 defect. It should be noted that the less restrictive clinical criteria for HNPCC (see Table I) identify many more patients suspected of having HNPCC compared to the Amsterdam criteria, but a smaller proportion of these patients ultimately prove to have inherited MMR defects.

Many other genes encoding proteins implicated in MMR have been considered as potential HNPCC genes. Of these, the most studied is MSH6. Defects in the MSH6 gene cause a partial loss of MMR due to the partial redundancy between MSH6 and MSH3. MSH6 mutations result in a large increase in the rate of accumulation of base substitution mutations but only modest increases in the rate of accumulation of single base frameshift mutations. Different studies have reported that perhaps up to 3% of HNPCC families can be accounted for by germline MSH6 defects, with these kindreds having a higher proportion of endometrial cancer than MSH2- or MLH1-detective HNPCC families. In contrast, approximately 8% of familial, non-HNPCC colorectal cancer cases were found to have germline MSH6 defects; similarly, germline MSH6 defects have been found in endometrial cancer cases associated with a weak family history of cancer. Such familial cancer cases constitute a much larger number of total cancer cases than HNPCC, indicating that germline defects in MSH6 could be almost as prevalent in the population as germline MSH2 and MLH1 defects. Compared to MSH2- or MLH1-defective HNPCC kindreds, MSH6-defective kindreds had a less striking family history and later age of onset of cancer, a result that parallels the results obtained in studies of MSH2-, MSH6-, and MLH1-defective mice. While more work on MSH6 is needed, it

**TABLE I**

**Clinical Criteria for HNPCC[a]**

| Name | Criteria |
|---|---|
| Amsterdam[b] | Three relatives with CRC, one of which must be a first-degree relative of the other two; CRC involving at least two generations of the family; one or more CRC cases diagnosed before age 50. |
| Modified Amsterdam[c] | 1. Small pedigrees, which cannot be further extended, can be considered as HNPCC if they contain CRCs in first-degree relatives; CRC must involve at least two generations and one or more CRC cases must be diagnosed before age 55.<br>2. In pedigrees where CRC is found in two first-degree relatives, a third relative with an unusually early-onset cancer or endometrial cancer is sufficient. |
| Young age of onset | A CRC case diagnosed at less than 40 years of age, without a family history fulfilling Amsterdam or modified Amsterdam criteria. |
| HNPCC variant | A family history of cancer suggestive of HNPCC, but not fulfilling Amsterdam, modified Amsterdam, or young age of onset criteria. |
| Bethesda[c] | 1. Individuals in families that fulfill Amsterdam criteria.<br>2. Individuals with two HNPCC-related cancers, including synchronous and metachronous CRCs or associated extracolonic cancers.<br>3. Individuals with CRC who have a first-degree relative with CRC and/or a HNPCC-related extracolonic cancer and/or colorectal adenoma; one of the cancers diagnosed before age 45 and the adenoma diagnosed before age 40.<br>4. Individuals with CRC or endometrial cancer diagnosed before age 45.<br>5. Individual with right-sided CRC with an undifferentiated pattern (solid/cribform) on histopathology diagnosed before age 45.<br>6. Individuals with signet-ring-cell-type CRC diagnosed before age 45.<br>7. Individuals with adenomas diagnosed before age 40. |

Adapted from Syngal S., Fox, E. A., Li, C., Dovidio, M., Eng, C., Kolodner, R. D., and Garber J. E., (1999). Interpretation of genetic test results for hereditary nonpolyposis colorectal cancer: implications for clinical predisposition testing. *J. Am. Med. Assoc.* **282**, 247–253.

[a] HNPCC, hereditary nonpolyposis colorectal carcinoma; CRC, colorectal cancer.

[b] All criteria must be met.

[c] Meeting all features under a single numbered criteria is sufficient.

appears that germline MSH6 defects cause an attenuated form of HNPCC.

A number of other MMR genes have been considered as possible HNPCC genes, including the MSH3 gene encoding a MutS homologue, the MutL homologue genes PMS1 (MLH2 in *S. cerevisiae*), PMS2 (PMS1 in *S. cerevisiae*) and MLH3, and the exonuclease encoding gene EXO1. However, defects in these genes have not been found to make a major contribution to HNPCC. This observation is consistent with many studies, indicating that defects in these genes do not cause complete loss of MMR because they encode partially redundant functions. It is also possible that these genes do not play roles in other MMR-related functions such as DNA damage-induced cell cycle arrest and apoptosis. Germline mutations in PMS2 appear to be only a rare cause of HNPCC, and most germline PMS2 mutations have been found to be associated with Turcot syndrome. In some sense this is surprising, because mutations in PMS2 cause strong MMR defects; however, PMS2-defective mice, while cancer prone, have a much different tumor spectrum compared to MSH2-, MSH6-, or MLH1-defective mice. Following the initial report of a germline PMS1 mutation, no other mutations have been reported, and recently the original PMS1 mutant family was shown to contain a MSH2 mutation. Mutations in MLH3 are also likely to be rare in HNPCC. Most studies have not identified germline MLH3 mutations in HNPCC cases, whereas the one study that did report potential mutations only identified missense variants that could be rare polymorphisms. Similarly, after an initial report of EXO1 mutations in HNPCC cases, a follow-up study showed that most of the initially reported variants, including a splice site mutation, were actually polymorphisms. This is consistent with the observation that EXO1 mutant mice show increased but much later onset

cancer susceptibility than MSH2 or MLH1 mutant mice. No germline defects in the MSH3 have been reported to date, although somatic mutations in MSH3 have been found as modifiers of germline MSH6 mutations consistent with studies of mutant yeast and mice. It is conceivable that defects in these genes could cause cancer susceptibility, although extrapolating from studies in model systems suggests such cancer susceptibility might be of later onset and weaker family history than HNPCC.

A variable proportion of sporadic cancers of many types has been observed to show tumor MSI indicative of a MMR defect. For example, in the case of sporadic colorectal cancer, the reported proportion of MSH-H tumors (note that MSI-H indicates a MMR defect) is around 18%. Most tumors showing MSI have been shown to lose expression of either MSH2 or MLH1, although the majority of cases show the loss of MLH1 expression. Most cases showing loss of MLH1 expression are due to somatic silencing of both copies of the MLH1 gene associated with hypermethylation of the MLH1 promoter. A small number of sporadic cases have been attributed to somatic mutations in MMR genes. These results indicate that there are both inherited and sporadic forms of MMR defective cancers, although the etiology of the two different types of cases is quite different.

How do MMR defects actually cause the development of cancer? Loss of MMR results in increased rates of accumulating mutations. This would be expected to increase the rate of accumulation of mutations that inactivate tumor suppressor genes and activate proto-oncogenes and consequently increase the rate of tumorigenesis. Considerable data exist that support this view. In addition, MMR-defective cells have checkpoint defects and are resistant to killing by DNA-damaging agents, suggesting that MMR defects also cause a defect in apoptosis that may also contribute to tumorigenesis. Because of this resistance to killing by DNA-damaging agents, MMR-defective tumors may also not be as responsive to some chemotherapeutic agents as non-MMR defective tumors.

## Summary

MMR requires two different heterodimeric complexes of MutS-related proteins, MSH2–MSH3 and MSH2–MSH6, that recognize mispaired bases in DNA. Two different heterodimeric complexes of MutL-related proteins, MLH1–PMS2 (*S. cerevisiae* PMS1) and MLH1–MLH3, also function in MMR and appear to interact with other MMR proteins, including the MSH complexes and replication factors. Additional proteins, including DNA polymerase delta, RPA, PCNA, RFC, and exonuclease 1, have been implicated in MMR. Loss of function of three of these genes, MSH2, MSH6, and MLH1, has been shown to be the cause of both hereditary cancer susceptibility syndromes and sporadic cancers, whereas mutations in other MMR genes have not yet been shown to be associated with a significant number of hereditary or sporadic cancer cases.

## See Also the Following Articles

DNA Mismatch Repair and Homologous Recombination • DNA Mismatch Repair and the DNA Damage Response • DNA Mismatch Repair: *E. coli* Vsr and Eukaryotic G–T Systems • DNA Mismatch Repair in Mammals • DNA Polymerase δ, Eukaryotic

## Glossary

**base substitution mutation** The class of mutations that result when an incorrect base is paired with the correct base that is present in the DNA.

**DNA polymerase** The class of enzymes that synthesize DNA using one DNA strand as a template to direct DNA synthesis.

**DNA replication** The process by which cells make an exact copy of their DNA.

**exonuclease** The class of enzymes that degrade DNA molecules starting from the ends of DNA or breaks in DNA strands.

**first-degree relative** A parent, sibling, or child of the individual in question.

**frameshift mutation** Type of mutation that results from either insertion or deletion of one or a small number of extra bases in DNA.

**germline mutation** Mutation that is transmitted through the germline (e.g., inherited mutation).

**heterodimer** Complex composed of two different proteins.

**immunohistochemistry (IHC)** Use of antibodies to stain tissue slices to detect the presence of the antigen (protein or other molecule to which the antibody binds).

**microsatellite instability (MSI)** The accumulation of frameshift mutations within the simple repeat sequences found ubiquitously in most eukaryotic chromosomal DNA.

**mispaired base** An incorrectly paired base in DNA, e.g., an A paired with a C instead of a T, or an extra base inserted into DNA so that it is not paired with any base.

**mutation** Change in the normal sequence of a DNA molecule.

**MutL** Bacterial MutL protein or gene. MutL is a protein that interacts with the MutS protein during bacterial MMR.

**MutS** Bacterial MutS protein or gene. Mut is an abbreviation for mutator, because inactivation of a mutator gene results in increased rates of accumulating mutations. MutS is the protein that recognizes mispaired bases in DNA during bacterial MMR.

**oncogene** Gene whose increased or altered activity promotes the development of tumors. Oncogenes often accumulate activating mutations or are amplified during tumor development.

**penetrance** The percent chance that a mutation carrier will develop the disease in the individual's lifetime.

**proliferating cell nuclear antigen (PCNA)** A protein that forms a ring around DNA and functions to keep proteins such as DNA polymerases attached to the DNA.

**redundancy** Two genes or proteins are redundant when they can substitute for each other.

**replication protein A (RPA)/replication factor A (RFA)** Protein complex that binds to single-strand DNA, allowing it to be a better template for DNA polymerases.

**replication factor C (RFC)** Protein complex required to load PCNA onto DNA.

**somatic mutation** Mutation that occurs in a somatic cell. Such mutations are not inherited.

**tumor suppressor gene** Gene whose product prevents the development of a tumor. Tumor suppressor genes accumulate inactivating mutations during tumor development.

## Further Reading

Fishel, R. (2001). The selection of mismatch repair defects in hereditary nonpolyposis colorectal cancer: Revising the mutator hypothesis. *Cancer Res.* **61**, 7369–7374.

Harfe, B. D., and Jinks-Robertson, S. (2000). DNA mismatch repair and genetic instability. *Annu. Rev. Genet.* **34**, 359–399.

Kolodner, R. D., and Marsischky, G. T. (1999). Eukaryotic DNA mismatch repair. *Curr. Opin. Genet. Dev.* **9**, 89–96.

Li, G.-M. (1999). The role of mismatch repair in DNA damage-induced apotosis. *Oncol. Res.* **11**, 393–400.

Loeb, L. A. (2001). A mutator phenotype in cancer. *Cancer Res.* **61**, 3230–3239.

Modrich, P., and Lahue, R. (1996). Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu. Rev. Biochem.* **65**, 101–133.

Nakagawa, T., Datta, A., and Kolodner, R. D. (1999). Multiple functions of MutS- and MutL-related heterocomplexes. *Proc. Natl. Acad. Sci. USA* **95**, 14186–14188.

Peltomake, P. (2003). Role of DNA mismatch repair in the pathogenesis of human cancer. *J. Clin. Oncol.* **21**, 1174–1179.

Sixma, T. K. (2001). DNA mismatch repair: MutS structure bound to mismatches. *Curr. Opin. Struct. Biol.* **11**, 47–52.

Wei, K., Kucherlapati, R., and Edelmann, W. (2002). Mouse models for human DNA mismatch-repair gene defects. *Trends Mol. Med.* **8**, 346–353.

## Biography

Richard D. Kolodner is Head of the Laboratory of Cancer Genetics at the Ludwig Institute, San Diego, and Professor, Department of Medicine and Department of Cellular and Molecular Medicine at the University of California at San Diego, School of Medicine. He is an Associate Editor of *Cell* and serves on the editorial board of *Cellular and Molecular Biology*, the *Journal of Biological Chemistry*, and *DNA Repair*. He has published more than 200 papers on DNA repair and recombination and received numerous awards for this work.

# DNA Mismatch Repair in Bacteria

**A-Lien Lu**
*University of Maryland, Baltimore, Maryland, USA*

**There are two major DNA mismatch repair (MMR) systems in bacteria to correct DNA biosynthetic errors. Long-patch repair systems involve a long-patch excision and resynthesis (up to 1000 nt) and are specific to a particular DNA strand. Short-patch repair systems have a repair tract shorter than 15 nt and are dictated by the nature of the mismatches. MMR enhances the fidelity of DNA replication and genetic recombination, and it participates in the cellular response to certain types of DNA damage. The inactivation of DNA repair systems can lead to genomewide instability and a predisposition to cancers in mammals. The importance of MMR in mutation avoidance is widely documented. Mutations in long-patch MMR genes result in a mutator phenotype with a spontaneous mutation frequency elevated approximately 100-fold. The mutation spectrum reveals that transitions and short nucleotide insertions/deletions predominant. MMR-defective strains are also hypersensitive to base-substitution mutagenesis and resistant to some types of alkylating agents. MMR also has an antirecombination function in limiting recombination between divergent sequences and preventing gross chromosomal rearrangement. MMR proteins have been shown to function in preventing interspecies recombination between *Escherichia coli* and *Salmonella typhimurium*.**

## *Escherichia coli* Long-Patch Mismatch Repair is Dependent of *dam* Methylation

For mismatch repair (MMR) to correct replication error, a strand-discrimination system must target the repair to the daughter DNA strand that contains the incorrect nucleotides. *E. coli* MMR is dependent on *dam* (DNA adenine methylation) methylation and is biased to unmethylated DNA strands. The *dam* methylase modifies the adenine at the 6-$NH_2$ group in GATC sequences. Wagner and Meselson proposed in 1976 that transient undermethylation of GATC sites in newly synthesized DNA provides the basis to direct the MMR to the daughter strands, an idea that has been confirmed both *in vivo* and *in vitro*. Because methylation is a postsynthetic process, methylation on the parental strands and transient unmethylation on the newly synthesized strands provide the signal to direct the repair to the daughter strands. MMR can be directed by hemimethylated sites located either 5′ or 3′ to the mismatch at a separation distance of up to 1 kb.

## Specificity of Mismatch Repair

Methyl-dependent MMR has a broad specificity. Of the eight possible base-base mismatches, only C–C is refractory to the repair. Heteroduplexes containing short insertion/deletion loops (IDL) derived from DNA polymerase slippage can also be repaired. IDL with three unpaired nucleotides are efficiently repaired, but IDL with four to seven unpaired nucleotides are poorly repaired. The efficiency of MMR is influenced by the sequence flanking the mismatch. The MMR system has also been shown to be involved in repair of oxidative DNA damage. The major oxidative lesion, 7,8-dihydro-8-oxo-guanine (8-oxoG), can mispair with adenine during DNA replication. *E. coli* MMR can also act on A-8-oxoG mismatches that result from replication errors.

## Mechanism of *E. coli* Long-Patch Mismatch Repair

An *in vitro* assay developed by Lu *et al.* has provided the basis for purifying and characterizing the *E. coli* MutH, MutL, and MutS proteins. Biochemical studies from P. Modrich's laboratory show that *E. coli* MMR involves 11 protein activities: MutS, MutL, MutH, UvrD (DNA helicase II), four single-strand specific exonucleases, a single-strand DNA binding protein (SSB), DNA polymerase III (Pol III) holoenzyme, and DNA ligase (Table I). Whereas MutH, MutL, and MutS proteins are specific for MMR, the other activities are involved in other DNA metabolic pathways, including replication and recombination. The mechanism of *E. coli* long-patch MMR is depicted in Figure 1.

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Functions of *E. coli* Mismatch Repair Enzymes**

| Enzyme | Function |
|---|---|
| MutS | DNA mismatch/damage recognition |
| MutL | Molecular matchmarker; Enhances mismatch recognition; stimulates MutH and UvrD activities |
| MutH | Strand discrimination; cleaves at unmethylated DNA strands |
| UvrD | DNA helicase II; unwinding DNA |
| ExoI, ExoX | 3′ to 5′ exonuclease; removes nucleotides |
| ExoVII, RecJ | 5′ to 3′ exonuclease; removes nucleotides |
| DNA Pol III holoenzyme | DNA resynthesis of the long-patch mismatch repair pathway |
| $\beta$ Sliding clamp | A subunit of DNA Pol III holoenzyme; interacts with MutS |
| SSB | Protecting DNA from degradation and re-anneal |
| DNA ligase | Nick ligation |
| MutY | DNA glycosylase; excises A from A/G, A/8-oxoG, A/C and G-8-oxoG mismatches and removes G from G/8-oxoG mismatches |
| Vsr | Endonuclease; removes T from T-G mismatch |
| DNA polymerase I | Repair synthesis of short-patch mismatch repair pathways |

## Initiation of Mismatch Repair

MMR requires specifically MutH, MutL, and MutS proteins in the initiation steps. MutS recognizes base–base mismatches and short insertion/deletion loops. MutL serves to couple mismatch recognition by MutS to activate several downstream activities, such as MutH and DNA helicase II. MutH endonuclease cleaves at the 5′ of G of an unmethylated GATC sequence. In a similar manner, MutS and MutL activate the excision process.

### MutS

MutS is a homodimer of a 95-kDa polypeptide that recognizes all base-base mismatches except C–C mismatch and small insertion/deletion loops up to five unpaired nucleotides. The discrimination of a mismatch-containing heteroduplex from a homoduplex by MutS protein is not great; there is only 10- to 20-fold preference. It has been suggested that mismatch recognition by MutS may be enhanced by MutL or by the association with replication proteins. MutS has ATPase activity, which is essential for the MMR function. ATP binding reduces the MutS affinity for heteroduplex DNA. Although several models have been proposed, the role of ATPase of MutS is still under investigation. The translocation and sliding clamp models suggest that MutS moves away from the mismatch site to activate MMR. The translocation model suggests that ATP hydrolysis provides the energy and enables MutS to move like a motor protein. This model is supported by a $\alpha$-loop-like DNA structure visualized under electron microscopy. The sliding clamp model or molecular switch model suggests that ATP-bound MutS slides along DNA without ATP hydrolysis. There is a conformation change from ADP-bound MutS to ATP-bound MutS. The third model suggests that the ATPase activity of MutS plays a proofreading role in MMR. The structure of MutS-DNA-ADP suggests that MutS has to bind both ATP and the mismatched DNA simultaneously to activate the repair process. Thus, the model suggests that MutS remains bound to the mismatch site and recruits MutL and MutH to initiate MMR.

The structure of *E. coli* MutS complexed with a G–T mismatch and *Thermus aquaticus* MutS with a + 1T IDL have been solved recently by the W. Yang and T. Sixma groups. Although different types of mismatches were used, both MutS structures are very similar. These structures show that a truncated form of MutS is a homodimer like an oval disk with two channels, one of which forms by domains I and IV and binds to the DNA. The bound DNA is kinked by 60° at the mismatch and the minor groove is widened and contacted extensively by MutS. Interestingly, MutS forms an asymmetric dimer with only one monomer making direct contact with the mismatched base. MutS contacts the mismatched bases through a highly conserved N-terminal motif Phe-X-Glu. The Phe residue in this conserved motif is inserted into the double helix and stacks with the mismatched thymine. The ATP binding site at the C terminus (domain V) of the protein is composed of residues from both subunits and is located far from the DNA-binding site. Domains II and III connect the mismatch-binding and ATPase domains. In the absence of DNA, domains I and IV are disordered in the crystal structure. DNA binding also alters the conformation of the ATPase domain.

### MutL

MutL is a dimer of a 68-kDa polypeptide that operates as a molecular matchmaker in MMR to assemble a functional repair complex. In the presence of ATP, MutL binds to the MutS-heteroduplex complex and activates MutH and UvrD helicase activities. The divergent C-terminal domain of MutL is responsible for the dimer formation. The conserved N-terminal domain has weak ATPase activity that is stimulated by DNA. Structural and biochemical studies indicate that ATP binding and hydrolysis modulate the conformation, oligomeric state of MutL, and interactions of MutL with MutS, MutH, and UvrD helicase. Upon ATP

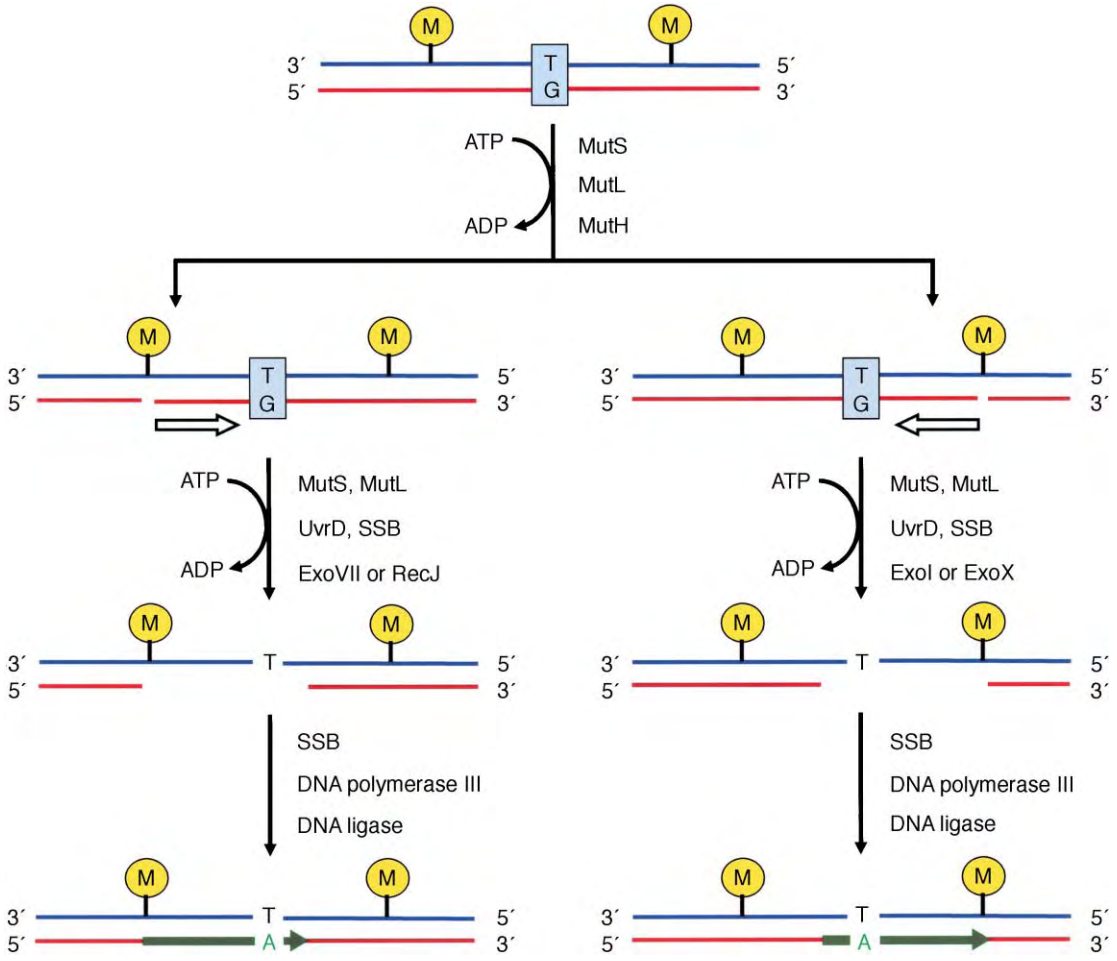



**FIGURE 1** The long-patch methyl-dependent mismatch repair system of *E. coli*. Mismatches (G–T, as shown) are recognized by dimeric MutS protein. After binding of MutL and MutH, the unmethylated strand in red is nicked by MutH at the hemimethylated GATC sites (M in yellow circle represents for methyl groups). ATP hydrolysis by MutS and MutL triggers the downstream repair process. DNA unwinding by UvrD (DNA helicase II) in the presence of ATP from the nick site toward the mismatch reveals a single-stranded DNA region that is protected by SSB. Depending on the position of the nick relative to the mismatch, ExoVII or RecJ with 5′ to 3′ exonuclease activity and ExoI or ExoX with 3′ to 5′ exonuclease activity degrade the nicked strand from the nick site up to and slightly past the mismatch. The resulting single-stranded gap undergoes repair resynthesis by the DNA polymerase III holoenzyme and the nick is finally sealed by DNA ligase. Open arrows represent the direction of exonuclease degradation and thick green arrows show the direction of repair resynthesis.

binding, the N-terminal domain undergoes conformation changes and dimerizes, although the native protein exists as a dimer in solution.

### MutH

The 25-kDa monomer MutH belongs to a family of type II restriction enzyme. MutH activity is activated by MutS and MutL in the presence of ATP and a mismatch-containing DNA. MutH cleaves at the 5′ side of the G of the GATC sequence on unmethylated strand located on either the 5′ or 3′ side of the mismatch. Tyr212 of MutH is important in sensing the methylation status of DNA. The structure of MutH is like a clamp with a cleft and two arms. DNA may be bound in the cleft.

## Excision and Repair Synthesis of Mismatch Repair

The strand break created by MutH at a GATC site of the unmethylated strand serves as the starting point for the excision of the mispaired base. UvrD (DNA helicase II) physically interacts with MutL and its unwinding activity is dramatically stimulated by MutL. MutL facilitates the loading of helicase II on the appropriate strand to unwind DNA in the proper direction and allows repeated loading of additional helicase II molecules to continue the unwinding reaction. DNA helicase II unwinds the DNA from the nick toward the mismatch and reveals a single-stranded DNA region that is protected by SSB.

The excision of the displaced strand is then initiated from the nick by exonucleases and is dependent on MutS, MutL, and UvrD helicase. Depending on the position of the nick relative to the mismatch, ExoVII or RecJ with 5′ to 3′ exonuclease activity and ExoI or ExoX with 3′ to 5′ exonuclease activity degrade that portion of the nicked strand displaced by the helicase up to and slightly past the mismatch. The resulting single-stranded gap is repaired by the DNA polymerase III holoenzyme, and the nick is finally sealed by DNA ligase. MMR may be coupled with DNA replication via physical interaction between MutS and the $\beta$-clamp of DNA polymerase III holoenzyme.

## MutY Repair Pathway

In addition to the long-patch methyl-dependent MMR, two short-patch repair pathways have been characterized in *E. coli*. Only the MutY repair pathway is described here and the very short patch (VSP) pathway initiated by Vsr endonuclease is covered elsewhere in this encyclopedia.

Oxidative damage is a major source of mutation load in living organisms. 8-OxoG is one of the most stable products of oxidative DNA damage and has the most deleterious effects because it can mispair with adenine. In *E. coli*, MutT, MutS, MutM, and MutY are involved in defending against the mutagenic effects of 8-oxoG lesions (Figure 2). The MutT protein eliminates 8-oxo-dGTP from the nucleotide pool with its pyrophosphohydrolase activity, whereas the MutM glycosylase (Fpg protein) removes both mutagenic 8-oxoG adducts and ring-opened purine lesions. MutS and MutY increase replication fidelity by removing the adenines misincorporated opposite 8-oxoG or G during DNA replication. The *E. coli* MutY protein specifically removes mispaired adenines from A–G, A-8-oxoG, and A-C mismatches and removes guanines from G-8-oxoG mismatches. A-8-oxoG mismatches are particularly important biological substrates for MutY. The mutY mutants have approximately 50-fold higher mutation frequencies of G–C to T–A transversions than wild-type cells.

MutY is a 39-kDa DNA glycosylase that removes a free base from DNA to initiate a base excision repair (BER) process. The MutY BER pathway involves DNA polymerase I and DNA ligase and may involve apurinic–apyrimidinic (AP) endonuclease (EndoIV or ExoIII). Both AP endonucleases enhance the product release with A–G but not A–8-oxoG substrates. Although MutY can form a covalent Schiff base intermediate with its DNA substrates, it is controversial whether MutY has AP lyase ($\beta$-elimination) activity in addition to the DNA glycosylase activity. It has been suggested that coupling to DNA replication ensures that MutY BER is targeted to the daughter DNA strands but not the parental strands.

MutY glycosylase contains a [4Fe–4S] iron–sulfur cluster and it belongs to a conserved helix–hairpin–helix (HhH) family that includes several DNA glycosylases.

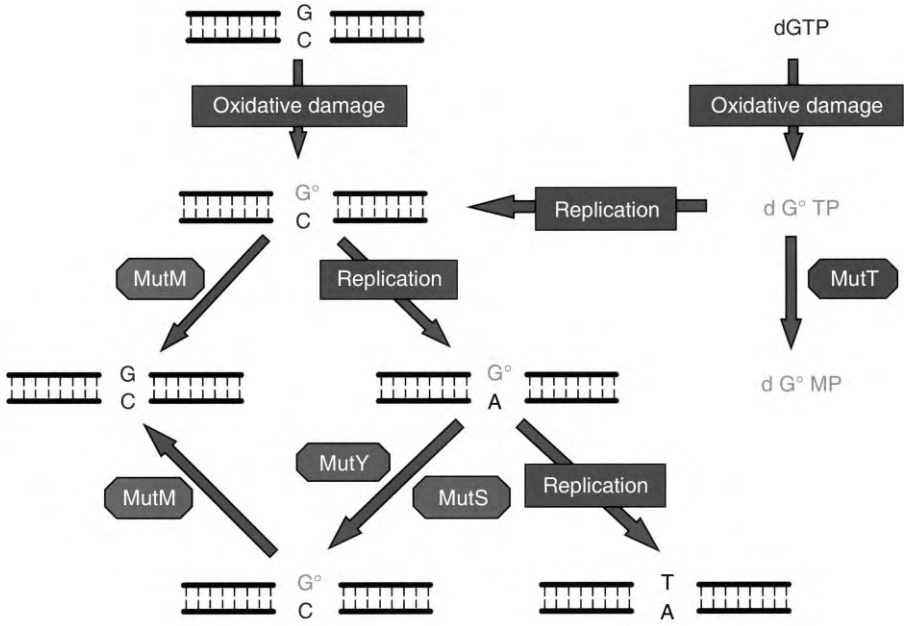



**FIGURE 2** 8-OxoG repair in *E. coli*. The MutT protein hydrolyzes 8-oxo-dGTP (dG°TP) to 8-oxo-dGMP (dG°MP) and pyrophosphate. The MutM glycosylase (Fpg protein) removes mutagenic 8-oxoG (G°) adducts while they are paired with cytosines. When C–8-oxoG is not repaired by MutM, adenines are frequently incorporated to the 8-oxoG bases during DNA replication. MutS and MutY increase replication fidelity by removing the adenines misincorporated opposite 8-oxoG.

The X-ray crystal structure of the catalytic domain of D138N-MutY with bound adenine shows that adenine is buried in the active site of the catalytic domain, suggesting that the mismatched adenine must flip out of the DNA helix for glycosylase action. The iron–sulfur cluster loop is important in substrate recognition and MutY stability, and the HhH motif is involved in binding to the phosphate backbone. The structure of MutY from *Bacillus stearothermophilus* in complex with A-8-oxoG-containing DNA was recently determined. Similar to several DNA glycosylases, MutY distorts the bound DNA and flips out the mismatched adenine out of the helix but the mismatched GO remains intrahelical. The C-terminal domain of MutY has been shown to play an important role in the recognition of GO lesions. The C-terminal domain of MutY has no homology to other members of the HhH superfamily, but shares some sequence and structural similarities to MutT.

## See Also the Following Articles

DNA Base Excision Repair • DNA Helicases: Dimeric Enzyme Action • DNA Ligases: Mechanism and Functions • DNA Ligases: Structures • DNA Mismatch Repair and Homologous Recombination • DNA Mismatch Repair: *E. coli* Vsr and Eukaryotic G–T Systems • DNA Polymerase III, Bacterial • Recombination: Heteroduplex and Mismatch Repair *in vitro*

## Glossary

**base excision repair (BER)** A DNA repair pathway that is initiated by a DNA glycosylase to remove relatively small lesions. Some glycosylases are highly specific to certain lesions or mismatches.

***dam* methylation** The methylation of the adenine at the 6-NH2 group in GATC sequences by *dam* methylase. Methylation on the parental strands and transient unmethylation on the newly synthesized strands provide a signal to direct the mismatch repair to the daughter strands.

**mismatch repair (MMR)** Process in which the long-patch MMR removes replicative errors that are missed by DNA polymerase's proofreading activity. MMR also functions during genetic recombination and corrects certain types of DNA damage.

**mutator phenotype** A phenotype that is associated with elevated genomewide spontaneous mutation frequencies and is usually caused by mutations at the DNA repair genes.

**replication fidelity** The extremely low error rate of DNA replication, which is achieved by the accuracy of DNA replication and mismatch repair.

## Further Reading

Friedberg, E. C., Walker, G. C., and Siede, W. (1995). *DNA Repair and Mutagenesis*. ASM Press, Washington, D.C.

Hsieh, P. (2001). Molecular mechanisms of DNA mismatch repair. *Mutat. Res.* **486**, 71–87.

Lindahl, T., and Wood, R. D. (1999). Quality control by DNA repair. *Science* **286**, 1897–1905.

Lu, A.-L., Li, X., Gu, Y., Wright, P. M., and Chang, D.-Y. (2001). Repair of oxidative DNA damage. *Cell Biochem. Biophys.* **35**, 141–170.

Modrich, P. (1991). Mechanisms and biological effects of mismatch repair. *Annu. Rev. Genet.* **25**, 229–253.

Modrich, P., and Lahue, R. (1996). Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu. Rev. Biochem.* **65**, 101–133.

Yang, W. (2000). Structure and function of mismatch repair proteins. *Mutat. Res.* **460**, 245–256.

## Biography

A-Lien Lu is a Professor in the Department of Biochemistry and Molecular Biology at the University of Maryland, Baltimore. Her principal research interest is in DNA repair, including long-patch MMR and short-patch MutY repair. She holds a Ph.D. from the University of North Carolina at Chapel Hill and received her postdoctoral training at Duke University. As a postdoctoral fellow, she developed an *in vitro* MMR assay that has been used to purify and characterize MMR proteins, and her laboratory was the first to discover the *E. coli dam*-independent MutY pathway.

# DNA Mismatch Repair in Mammals

**James T. Drummond**
*Indiana University, Bloomington, Indiana, USA*

**When DNA polymerases copy an organism's genomic DNA, errors that escape the replication complex appear as mismatches. Virtually every mispairing may be recognized and corrected by a set of activities known collectively as the mismatch repair (MMR) pathway. When this pathway is disabled in humans, either via mutation or epigenetic silencing, the mutation rate rises approximately 100- to 1000-fold. There is a generally predictable outcome when dividing cells become mutators; deleterious changes within critical genes that regulate cell growth or suppress tumors result in a clonal expansion that fuels a cycle of further mutation and selection for phenotypes that spur growth or avoid death. Such a sequence describes tumors in hereditary cancer syndromes such as Muir–Torre and hereditary nonpolyposis colorectal cancer (HNPCC), and it frequently can explain sporadic tumor initiation in many tissue types.**

## Historical Perspective and Overview

Our understanding of the contributions of the DNA MMR pathway to the long-term well being of humans has exploded over the past decade. Substantial literature describing repair processes in model organisms such as bacteria and yeast has provided an indispensable road map for identifying mammalian homologs involved in tumor initiation and progression, beginning with the identification of human *MSH2* (*MutS Homolog 2*) in 1993. It quickly became apparent that the two primary genetic loci defective in HNPCC families corresponded to human *MSH2* and *MLH1* (*MutL homolog 1*), homologs of proteins essential for repairing mismatches in most complex organisms. The cast of participants rapidly evolved with revelations that MSH2 and MLH1 were isolated as heterodimeric species with distinct MutS and MutL homologs, each with different but often overlapping mechanistic contributions. In contrast to DNA repair pathways that remove site-specific lesions, repair of mismatches depends on identifying both the mismatch and a remote site that allows discrimination between parent and daughter strand. Large tracts of DNA may therefore be excised and resynthesized during a repair event. The replication machinery that restores the excised DNA sequence also participates in genomic replication, adding these proteins to the list of participants. Many of the proteins essential for DNA replication were found to have distinct roles in mismatch correction, most prominently PCNA (identified as a proliferating cell nuclear antigen). Despite the many advances, neither the full cast of players nor the specific mechanism for mismatch correction is fully characterized.

## Mechanistic Contributions of the Key Participants in Mammalian MMR

### MutS and Some of Its Homologs Recognize Mismatches

During a search for genetic defects that conferred a *Mut*ator phenotype in *E. coli*, inactivation of the *MutS* gene resulted in roughly a 1000-fold increase in the mutation rate. The MutS protein proved to be a mismatch-binding homodimer composed of 90 kD monomers with an inherent weak ATPase activity that plays an essential but incompletely defined role in mismatch correction. In mammals, the corresponding protein activities are more complex (Figure 1). The MSH2 protein forms tight complexes with either MSH6 or MSH3 (not shown); defects in *MSH2* apparently eliminate all nuclear MMR activity and increase the mutation rate from 100- to 1000-fold. Partnered with MSH6, MSH2 forms a heterodimer called MutS$\alpha$ that can recognize base–base and many small insertion-deletion mismatches. The MSH2/MSH3 heterodimer (MutS$\alpha$) is present in substantially lower amounts in tumor cells, and it recognizes primarily insertion–deletion mutations in which two or more unpaired nucleotides are present in either strand. These two mismatch-binding heterodimers provide partially overlapping mismatch recognition capabilities, and defects in either *MSH6* or *MSH3* are less severe mutators than *MSH2*. Each individual defect confers a mutation

 © 2004, Elsevier Inc. All Rights Reserved. 

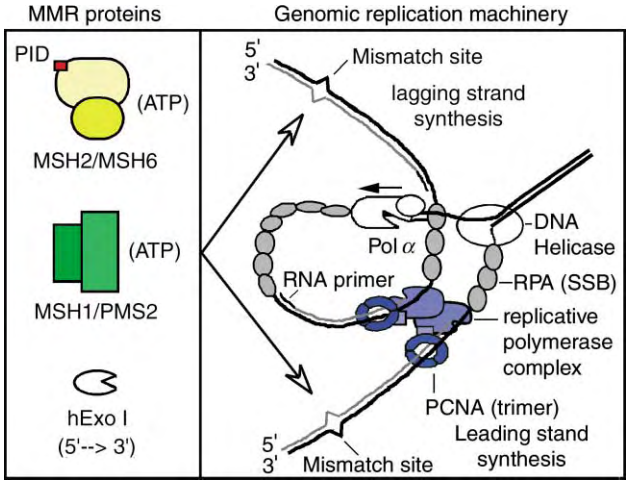



**FIGURE 1** MMR activities cooperate with the DNA replication apparatus. The box on the left shows the proteins specific to mismatch correction. DNA replication is illustrated on the right as a process where leading and lagging strand synthesis is coordinated. Mismatches, identified as inverted triangles, are corrected such that the daughter strand (light gray) is targeted specifically for excision and removal. Resynthesis restores the parental strand information (darker strand). Several key proteins in replication, such as PCNA, RPA (a single-stranded binding protein complex), and polymerase delta, are also required for mismatch correction.

spectrum consistent with the selective failure to recognize different classes of mutations.

While the MSH6 and MSH3 proteins form heterodimers with MSH2 that are involved in recognizing mismatches, other MutS homologs have distinct roles in DNA metabolism. The mammalian MSH4 and MSH5 proteins also heterodimerize, yielding a complex that participates in meiotic recombination. While the precise mechanistic contribution of this complex is not known, it does not appear to recognize DNA mismatches in an error avoidance pathway. One further MutS homolog identified in yeast that might be expected in human cells, MSH1, has not surfaced. This mitochondrial protein is known to recognize DNA mismatches and stabilize the yeast mitochondrial genome by an undefined mechanism.

### MutL and its Homologs are Essential to MMR

In prokaryotes such as *E. coli*, loss-of-function mutations in *Mut* L confer up to a 1000-fold increase in the mutation rate, much like defects in the *MutS* homologs. In parallel with MutS homologs, mammalian MutL homologs form heterodimers in which the MLH1 protein is central to complexes active in nuclear mismatch correction (Figure 1). MLH1 forms the primary heterodimer with PMS2 (PMS1 in yeast), which is also a *Mut* L homolog.

The mechanistic contribution of the MutL homolog heterodimers to repair, let alone other processes, is one of the least well-understood aspects of MMR. Some clues to MutL function are available from studies using yeast or *E. coli* proteins, but very little is known of their mammalian counterparts. Some MutL homologs are known to bind and utilize ATP, which is essential for mismatch correction. The nucleotide-binding motif is most similar to those of the growing category of $AAA^+$ proteins, which carry out diverse roles but often utilize ATP to drive dissociation of protein–protein or protein–DNA complexes. The purified human MutS$\alpha$ and MutL$\alpha$ heterodimers, as well as homologs from model organisms, have been shown to interact on longer DNA templates in the presence of ATP. This has led to models that suggest migration of a complex of both heterodimers along the DNA helix as part of the repair mechanism.

### PCNA and the Replication Apparatus

When MutS$\alpha$ recognizes mismatches and triggers a strand-specific excision event, hundreds to thousands of nucleotides may be removed. A processive, PCNA-dependent polymerase is required to fill in the gap, which in human cells has been identified as polymerase delta. In Figure 1, the replicative polymerase complex shown synthesizing the daughter strand contains polymerase delta, and the MMR and replication functions are likely to be closely linked. A central player shared by both processes is the PCNA molecule, which forms a trimeric sliding clamp for replication and plays a complex in mismatch correction. It is essential for the MMR reaction prior to strand excision, and it interacts with MSH2 and MSH6 via a small, conserved domain through which many other repair proteins also bind PCNA (labeled PID for *P*CNA *I*nteracting *D*omain in Figure 1). Consistent with the biochemical and genetic evidence for this interaction, the key MMR proteins can be identified within replication foci *in situ*. Precisely how MMR is coupled to replication, or how the processes are coordinated, remains poorly understood.

## Orchestration of Mismatch Correction in Mammals

The mechanism through which a DNA mismatch is repaired remains an intensely investigated field of research. Based on a wealth of genetic and biochemical data generated in model organisms, we have a clear idea of several participants and mechanistic intermediates along the pathway. How repair events are coordinated,

and the precise role of each of the players, remains unknown or a matter of dispute. Therefore, a series of intermediates in the process in which the proteins involved or the DNA structure is known, but for which the understanding of the mechanism remains incomplete, will be presented (Figure 2).

## How Does Mismatch Binding Trigger Repair?

To be corrected, a mismatched site needs to be distinguished from correctly paired DNA. While no crystal structure is available for any eukaryotic heterodimer, prokaryotic MutS homodimers (from *E. coli* and *Thermus aquaticus*) have been crystallized complexed with either base–base or base–insertion mismatches. These structures reveal that mismatch recognition depends on the ability of MutS to kink the DNA helix sharply at the mismatch site by approximately 60°. The bend is partially enforced by the insertion of a phenylalanine side chain that stacks within the helix at the mismatch site. The MutS protein contains a domain that is poorly structured in the absence of DNA, and binding to the mismatch site orders the protein fold and sandwiches the DNA between the two halves of the homodimer. It has also been shown that eukaryotic MutS heterodimers are also capable of bending DNA upon mismatch binding.

As described above, MutS$\alpha$ in mammals is an ATPase, and it is clear that ATP triggers a conformational change that modifies DNA binding at the mismatch site. Precisely how that occurs, or which other protein activities might be involved *in vivo* (such as PCNA or MutL$\alpha$), remains to be established. The three competing models that address this question each have clear experimental support or mechanistic plausibility, yet none provide a satisfactory overall answer.

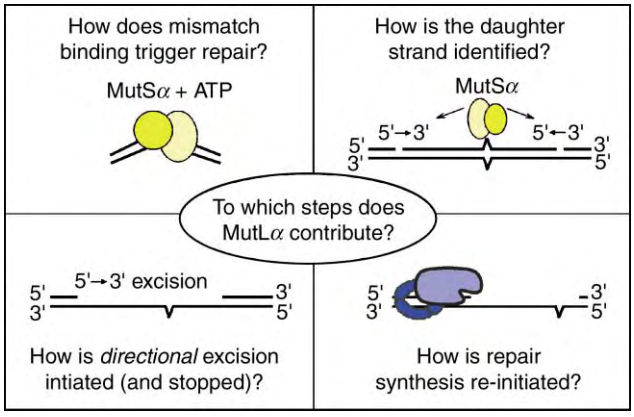



**FIGURE 2** Five prominent unanswered questions about human MMR. Note that the MMR pathway is bidirectional, and that the nick shown to the right of MutS$\alpha$ in the upper right box could also have been accessed to trigger a 3′ to 5′ excision event toward the mismatch.

Mechanisms that suggest ATP-dependent DNA looping provide an elegant solution for how bidirectional scanning for a repair-directing signal may occur. Mechanisms that suggest diffusion-controlled migration along the DNA helix are plausible and well supported, but they require multiple copies of MutS$\alpha$ *in vivo* to drive bidirectional scanning. Neither of these models provides a memory for the mismatch site, since the protein that originally binds the mismatch leaves the site and travels hundreds of base pairs away. A third model suggests that MutS$\alpha$ might stay associated with the mismatch site and communicate through space with a marker for the daughter strand, such as with PCNA within the replication complex (see Figure 1). This allows for an anchor at the mismatch site, but it fails to provide an explanation for how directional excision is initiated.

## How Is the Daughter Strand Identified?

Once a mismatch has been identified, the mandate of the MMR pathway is to target the newly synthesized DNA strand for excision and resynthesis. In a small group of prokaryotes that includes *E. coli*, the methylation status at GATC sequences reveals the daughter strand. The transient lack of methylation allows the MutH protein, stimulated by MutS and MutL at a nearby mismatch, to nick the daughter strand. However, such a methylation-based strategy apparently does not operate in mammalian cells. Instead, the most plausible mechanism to date suggests that mismatch correction is coupled with replication. By definition, the replication machinery generates the daughter strand, yielding a strand discrimination mechanism if the DNA end where synthesis occurs may be accessed.

## How is Directional Excision Initiated and Controlled?

One of the hallmarks of MMR in all organisms is a bidirectional capability. In other words, the information used to distinguish DNA strands may be either "upstream" or "downstream" with respect to the mismatch (Figure 2). This capability demands that any mechanism to explain mismatch correction must account for identifying nearby signals in both directions along the DNA helix simultaneously. Once a strand signal is identified, several further constraints are introduced. The system must identify the relative orientation between the mismatch site and the point of excision, then correctly initiate an excision event that moves toward and through the mismatch site. This requires competence for degrading the intervening DNA by correctly accessing an exonuclease with either a

5′ → 3′ or a 3′ → 5′ strand polarity. It has been shown that the human Exonuclease I (Exo I) is essential for repair with either strand polarity, but only a 5′ → 3′ excision capability has been demonstrated. Note that for clarity, only a 5′ to 3′ excision reaction is illustrated in Figure 2.

## How is Repair Synthesis Reinitiated?

Once DNA excision with either polarity is initiated, the replicative polymerase machinery must bring closure to it. It is clear that excision proceeds to relatively random sites approximately 150 nucleotides beyond the mismatch, with a wide variance and a few favored stopping points. Given that the single-strand excision event could span a few hundred to over a thousand base pairs, the MMR pathway must have a mechanism to ensure that excision proceeds until the mismatch site is removed. Once excision gives way to resynthesis, a new logistical problem is now apparent – if excision proceeds 5′ → 3′, the gap-filling polymerase (delta) has access to the DNA priming end from which the excision was initiated. In principle, resynthesis could track the excision process, leaving little or no gapped DNA as an intermediate. In the case of 3′ → 5′ excision, the entire excision event must be completed before the priming strand may be revealed to the polymerase. A long DNA gap is a required intermediate in this case. The repair process is formally concluded by the action of a DNA ligase that seals the nick that remains after resynthesis.

In summary, the MMR machinery is a complex assembly of proteins that can recognize DNA mismatches and restore the original DNA sequence. Failure of this pathway results in an increased mutational load and relaxed oversight of recombination events, wherein mismatches are also generated when the participating DNA strands are not identical. Such events are associated with tumorigenesis, revealing the crucial role of the MMR pathway in maintaining the genome of complex organisms under normal circumstances.

## See Also the Following Articles

DNA Mismatch Repair and Homologous Recombination • DNA Mismatch Repair Defects and Cancer • DNA Polymerase δ, Eukaryotic

## Glossary

**exonuclease** Enzyme that hydrolyzes nucleic acid polymers, usually to nucleotide monophosphates or short oligonucleotides, in a reaction initiated from a single- or double-stranded terminus.

**mismatch** Any pairing of DNA bases not described by the classical Watson–Crick G–C or A–T pairing, including addition or deletion of up to ~12 nucleotides in one DNA strand.

**mutation** Alternations in DNA sequence that result from processes such as unrepaired replication errors or DNA damage, which may or may not result in detectable phenotypic changes.

**PCNA** Proliferating cell nuclear antigen, a trimeric protein that forms a ring that can be pried open and loaded on double-stranded DNA to act as a platform for processive DNA synthesis or DNA repair activities.

## Further Reading

Bellacosa, A. (2001). Functional interactions and signaling properties of mammalian DNA mismatch repair proteins. *Cell Death Different.* **8**(11), 1076–1092.

Hsieh, P. (2001). Molecular mechanisms of DNA mismatch repair. *Mutat. Res.* **486**(2), 71–87.

Lynch, H. T., and de la Chapelle, A. (2003). Hereditary colorectal cancer. *N. Engl. J. Med.* **348**(10), 919–932.

Modrich, P. (1991). Mechanisms and biological effects of mismatch repair. *Annu. Rev. Genet.* **25**, 229–253.

Wei, K., Kucherlapati, R., and Edelmann, W. (2002). Mouse models for human DNA mismatch repair defects. *Trends Mol. Med.* **8**(7), 346–353.

## Biography

James T. Drummond is an Associate Professor of Biology at Indiana University in Bloomington, Indiana. He earned his doctoral degree in Biological Chemistry from the University of Michigan Medical School; he received his postdoctoral training at Duke University. His research interest in DNA metabolism is focused on two major goals: defining the mechanism through which human DNA mismatch repair pathway distinguishes the parent from the daughter strand, and how the pathway responds to lesions.

# DNA Mismatch Repair: *E. coli* Vsr and Eukaryotic G–T Systems

**Margaret Lieb**
*Keck School of Medicine, University of Southern California, Los Angeles, California, USA*

**5-Methylcytosine (5meC) is found at specific sites in the DNA of *Escherichia coli* and related bacteria, and also in eukaryotes, where it functions in the regulation of gene expression. Hydrolytic deamination of 5meC produces thymine (T) (Figure 1), which is mispaired with guanine (G) in the sister DNA strand. In *E. coli*, the very-short patch (VSP) repair system replaces the mismatched T with cytosine (C), thus preventing the occurrence of a C:G to T:A mutation during the next replication of the DNA (Figure 2). In the absence of VSP repair, spontaneous mutation at the sites of 5meC increases manyfold. VSP repair requires the specific endonuclease Vsr and also a DNA polymerase, pol I, and DNA ligase. MutS and MutL, proteins that are essential for the correction of mispairs arising during DNA replication, are required for efficient VSP repair. In mammals, 5meC occurs in CpG sequences. At least two different enzymes appear to preferentially excise T in a T:G mispair that arises in this context.**

## VSP Repair in *E. coli*

### Structure and Regulation of *vsr*

A small gene, *vsr*, coding for only 156 amino acids is present in *E. coli* and related enteric bacteria. It is located adjacent to *dcm*, whose product adds a methyl group to the second cytosine in a 5′ CC (A or T) GG sequence (Figure 2). The first seven codons of *vsr* overlap the 3′ end of *dcm* and both genes are transcribed from a single promoter. While their gene transcripts are present on the same mRNA, Dcm and Vsr proteins are expressed independently. In actively growing cultures, little Vsr is present in the bacteria, and mutation hot spots are found at the sites of 5meC. In nondividing bacteria, the concentration of Vsr increases significantly and, although deamination of 5meC continues at a rate that is time and temperature dependent, mutations do not accumulate at 5meC. Since the bacteria are expected to divide infrequently under natural conditions, VSP repair is an efficient mechanism to prevent mutation at 5meC.

### Biochemistry of Vsr

Vsr endonuclease is a monomeric protein whose overall structure and active site have similarities to type II restriction endonucleases. Vsr binds to DNA containing a T:G mispair resulting from the deamination of 5meC (Figure 2). It also binds, with lower affinity, to related sequences lacking the 5′C or the 3′G of the cognate sequence. Binding requires a divalent cation; however, magnesium but not calcium promotes subsequent Vsr activity. Crystallographic studies have shown that Vsr spreads apart the DNA strands at the mispair site by inserting amino acid side chains, causing the DNA to bend. *In vitro*, absence of 5meC from the strand opposite the mispaired T reduces both the rate of DNA binding and the efficiency of enzyme action. This effect has not been observed *in vivo*, where additional factors influence VSP repair.

Biochemical studies show that Vsr nicks DNA 5′ to the mismatched T. When large amounts of Vsr are present, the endonuclease activity does not require an additional protein. However, in replicating bacteria, VSP repair activity is enhanced significantly by MutS and MutL. Since the absence of either protein, or of both, has the same effect, it is likely that these products act cooperatively, as they are known to cooperate in mismatch correction of replication errors (MMR). MutS binds to T:G mispairs in any sequence context and recruits MutL resulting in a MutL–MutS complex that interacts with an endonuclease called MutH. Dominant negative mutations in MutS that block MMR also prevent VSP repair. Although the biochemistry has not been demonstrated *in vitro*, it is likely that MutS helps to attract Vsr to T:G mispairs, perhaps because Vsr can bind to MutL *in vitro*. If Vsr is brought to a T:G that is in the DNA sequence that it recognizes, it may cause MutS and MutL to disassociate from the mispair, allowing Vsr to replace MutS.

In addition to Vsr, VSP repair requires pol I, which has an intrinsic 5′–3′ exonuclease activity that digests away a small number of nucleotides starting at the nick immediately upstream of T. It simultaneously

 © 2004, Elsevier Inc. All Rights Reserved. 

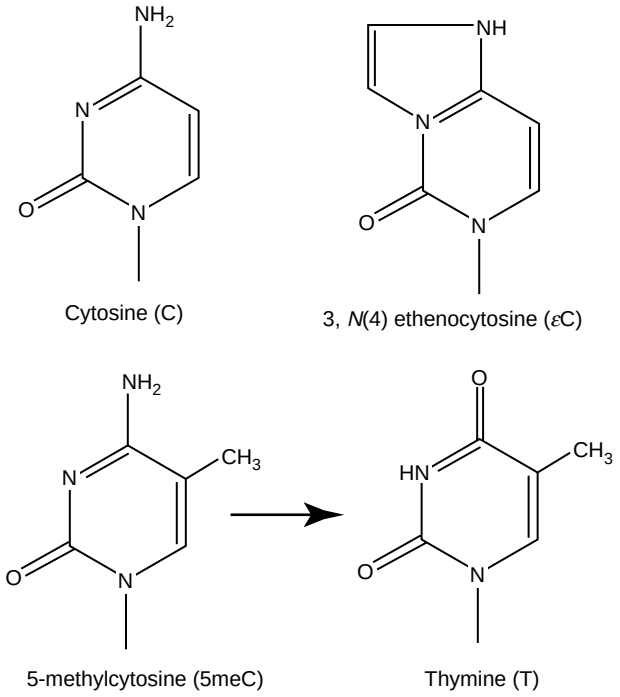



**FIGURE 1** Chemical structures of cytosine and cytosine derivatives. 3,N(4) ethenocytosine is a mutagen produced in DNA by endogenous products of lipid peroxidation and by industrial pollutants such as vinyl chloride.

resynthesizes DNA in the 5′–3′ direction. Since pol I is an error-free polymerase, it inserts a C in place of the T that it has removed from the mismatch. The actual number of bases removed is known to be less than 10, and is perhaps as few as 1 or 2. This is why this repair is referred to as very-short patch. The repair is completed by DNA ligase, an enzyme that seals single-strand nicks in double-stranded DNA.

### Mutagenesis by Vsr

Overproduction of Vsr increases the frequency of mutation at many sites in *E. coli*, mimicking the effect of mutations in *mutS* or *mutL*. Mutagenesis by Vsr can be attributed to its competition for a component of the MMR system. MutL has been shown to interact physically with Vsr *in vitro* and additional MutL reduces the mutagenic effect of excess Vsr. Thus, it is likely that the concentration of Vsr is maintained at a low level in dividing bacteria to prevent its titration of MutL. Vsr would also have a mutagenic effect if it corrected to C:G a T:G mispair in which the G was a replication error in a newly synthesized strand. It should be noted that MutS has a higher affinity for T:G than for any other mispair. Therefore, it is not surprising that excess MutS reduces VSP repair by competing with Vsr for binding at T:G mispairs that result from deamination of 5meC.

### VSP Repair and Marker Effects

Before the advent of DNA sequencing, the relative location of different mutation sites on microbial chromosomes was determined by crossing different mutants and observing the frequency of wild-type recombinants. The frequency of recombination generally decreases with the distance between mutation sites on the parental genomes. Certain mutations (or markers) appear to recombine with other mutations in their vicinity more frequently than expected. This "marker effect," which has also been referred to as "high negative interference," is often the result of VSP repair of a C to T mutation that occurred at a 5meC.

## Methyl-CpG Binding Domain 4 (MBD4) Protein in Eukaryotes

### Structure and Biochemistry of MBD4

The gene for MBD4 was identified in a search of the human sequence database for a methyl-CpG-binding domain. Mbd4 genes in both the human and mouse code for a 580 amino acid protein with a methyl-CpG-binding domain near the amino-terminal end, and a carboxy-terminal glycosylase domain. The glycosylase region of the protein is related to bacterial glycosylases such as MutY. The enzyme has no lyase activity, so that the removal of the mismatched base

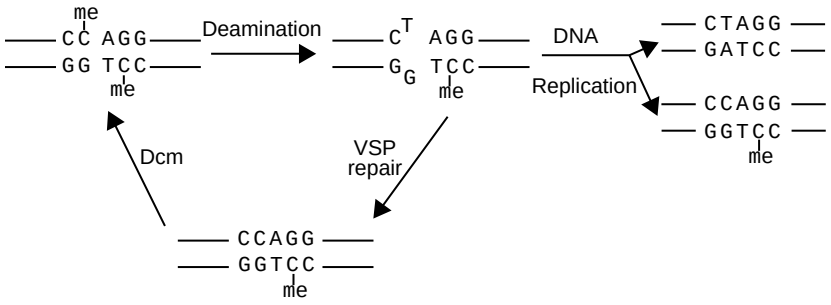



**FIGURE 2** The life cycle of a dcm site in *E. coli*.

leaves an abasic site. The sugar-phosphate backbone of DNA can subsequently be cleaved by the human AP endonuclease HAP1 but other endonucleases may also perform this function. There is evidence suggesting that processing of the abasic site also involves interaction with MLH1, a homologue of bacterial MutL.

### Specificity

Investigations with human MBD4 have shown that only T:G and U:G mispairs are good substrates for the enzyme. The human MBD4 enzyme binds preferentially to T:G mismatches in the meCpG/TpG context. However, removal of the mismatched base was observed when U:G and T:G mismatches were in a nonmethylated substrate. T:G mismatches in other contexts are processed at a greatly reduced rate. The fact that U is removed from a mispair with G more rapidly than T suggests that removal of T resulting from deamination of meC may not be the primary function of MBD4.

### Cancer Prevention

In the cells of the intestinal mucosa of mice containing inactive *Mbd4* genes there was a two- to threefold increase in C to T mutations in CpG sequences. In combination with mutations in the adenomatosis polyposis (Apc) genes, MBD4 inactivation increased the number of tumors and tumor progression. A 3.3-fold increase of C to T mutations at 5meC was also observed in the liver and spleen of mice lacking MBD4. Therefore, it is clear that MBD4 is a significant factor in the prevention of mutations leading to cancer.

## Thymine-DNA Glycosylase (TDG)

### Structure and Biochemistry of TDG

TDG is an enzyme with homologues found in the cells of humans and other animals, and also in *E. coli*. It has no apparent amino acid similarity to MBD4.The enzyme is characterized by low abundance and slow action. TDG can remove from DNA the bases T or U that arise by deamination of 5meC or C, and are thus mispaired with G. Removal of the base from the DNA backbone results in an abasic site to which the TDG remains bound. Studies of human TDG *in vitro* have indicated that an apurinic exonuclease (HAP1) helps TDG turnover and cleaves the abasic sugar phosphate. Repair of the resulting nick in the DNA strand requires both a DNA polymerase and DNA ligase.

### Specificity

TDG was first isolated in a search for a Vsr-like activity in extracts of human cells. The enzyme has a higher affinity for U:G mispairs than for T:G, and also significant affinity for 3,N(4) ethenocytosine (Figure 1) paired with G. TDG has a strong preference for mispairs arising in the CpG context. However, there is as yet no direct evidence that it has an important role in preventing mutation resulting from deamination of 5meC.

### See Also the Following Articles

DNA Mismatch Repair Defects and Cancer • DNA Mismatch Repair in Bacteria • DNA Restriction and Modification: Type II Enzymes • Recombination: Heteroduplex and Mismatch Repair *in vitro*

### Glossary

**AP endonuclease** An enzyme that makes incisions in DNA on the 5′ side of either apurinic or apyrimidinic sites.

**deamination** Hydrolytic replacement of an amino group ($-NH_2$) from a chemical compound with a hydroxyl group.

**dominant negative mutation** A mutation that results in a protein that is inactive and, in addition, competes with an active form of the same protein.

**glycosylase (glycosidase)** A hydrolytic enzyme that cleaves the bond between a sugar molecule and a nucleic acid base such as thymine.

**monomeric enzyme** An enzyme composed of a single polypeptide.

**type II restriction endonuclease** An enzyme that cuts both DNA strands at defined positions close to or within a specific base sequence.

### Further Reading

Bellacosa, A. (2001). Role of MED1(MBD4) gene in DNA repair and human cancer. *J. Cell Physiol.* **178**, 137–144.

Bhagwat, A. S., and Lieb, M. (2002). Cooperation and competition in mismatch repair: Very short patch repair and methyl-directed mismatch repair in *Escherichia coli*. *Mol. Microbiol.* **44**, 1421–1428.

Hardeland, U., Bentel, M., Lettieri, T., Jiricny, J., and Schar, P. (2003). The versatile thymine DNA glycosylase: A comparative characterization of the human, Drosophila and fission yeast orthologs. *Nucleic Acids Res.* **312**, 261–271.

Lieb, M., and Bhagwat, A. S. (1996). Very short patch repair: Reducing the cost of cytosine methylation. *Mol. Microbiol.* **20**, 467–473.

Miller, C. B., Guy, J., Sansom, O. J., Selfridge, J., MacDougall, E., Hendrich, B., Keightley, P. D., Bishop, S. M., Clarke, A. R., and Bird, B. (2002). Enhanced CpG mutability and tumorigenesis in MBD4-deficient mice. *Science* **297**, 403–405.

Wu, P., Qiu, C., Sohail, A., Zhang, X., Bhagwat, A. S., and Cheng, X. (2003). Mismatch repair in methylated DNA. *J. Biol. Chem.* **278**, 5285–5291.

### Biography

Margaret Lieb is Professor Emerita in the Department of Molecular Microbiology and Immunology at the Keck School of Medicine in Los Angeles. She holds degrees from Smith College, Indiana University and Columbia University, and pursued postdoctoral studies at Caltech and the Pasteur Institute. Her research on bacteriophage lambda genetics has focused on the lambda repressor. VSP repair was discovered in a study of recombination between mutations in the repressor gene.

# DNA Oxidation

**Arthur P. Grollman**
*State University of New York, Stony Brook, New York, USA*

**Dmitry O. Zharkov**
*Novosibirsk Institute of Chemical Biology and Fundamental Medicine, Russia*

**Oxygen-releasing photosynthesis and aerobic respiration have provided the basic machinery of life for almost three billion years and have driven the evolution of complex life forms. Molecular oxygen is a powerful oxidizing agent whose metabolic products can cause serious damage to proteins and DNA. As the integrity of genomic DNA is critical to cell survival, a variety of intracellular systems have evolved to repair oxidative DNA damage.**

## Oxidants and Antioxidants

The reduction of oxygen to water consists of several sequential steps involving the reactive oxygen species (ROS) superoxide anion radical, hydrogen peroxide, and hydroxyl radical. Thus,

$$O_2 \rightarrow {}^{\cdot}O_2^- \rightarrow H_2O_2 \rightarrow HO^{\cdot} \rightarrow H_2O$$

Additionally, molecular oxygen in the $^1\Delta_g$ excitation state ("singlet oxygen") is included in the category of reactive oxygen species. In cells, ROS react with many molecules, with their average lifetime and distance of diffusion from the point of generation being inversely proportional to their reactivity. Free radicals, such as ${}^{\cdot}O_2^-$ and $HO^{\cdot}$, trigger chain reactions of oxidation, allowing unpaired electrons to propagate over longer distances. Polyunsaturated fatty-acid side chains are especially prone to reactions with free radicals; the resulting peroxyl radicals propagate by a chain reaction. Given the ubiquitous presence of lipid bilayers in cells, lipid peroxidation also plays an important role in the toxicity associated with ROS.

The primary source of ROS in eukaryotes is the mitochondrial respiratory chain. Approximately 0.1% of cellular oxygen is converted to superoxide, primarily through auto-oxidation of the semiquinone forms of ubiquinone and flavin mononucleotides, which occurs during the NADH dehydrogenase electron transfer process. In mitochondria, superoxide anions generate hydrogen peroxide and hydroxyl radicals. In plants, photosystem I is also a major producer of ROS. Other cellular redox systems also are capable of producing ROS. For example, nonmitochondrial endogenous sources of ROS include reactions catalyzed by cytochrome P450, NADPH:cytochrome P450 reductase, and xanthine oxidase. Recently, immunoglobulins were identified as a major ROS source in mammals.

The major environmental source of ROS is ionizing radiation. Radiolysis of water releases hydroxyl radicals and other reactive entities, such as solvated electrons and ${}^{\cdot}H$ and ${}^{\cdot}O^-$ radicals. Other sources of ROS include microwave radiation, ultrasound, photosensitizing dyes (methylene blue, rose bengal), transition metal ions, ozone, and a variety of salts (e.g., $KBrO_3$), as well as certain drugs and chemicals.

Hydroxyl radicals and singlet oxygen are the most reactive forms of ROS and, consequently, the most damaging to biomolecules. Hydroxyl radicals are generated from superoxide and $H_2O_2$ by Fenton chemistry when $Fe^{2+}$ or other low-valency transition metal ions are present; such ions bind to DNA and these reactions play an important role in DNA damage:

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + OH^- + Fe^{3+}$$
$${}^{\cdot}O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

In living organisms, various mechanisms have evolved to minimize the effects of oxidative damage, including deactivation of ROS by superoxide dismutases, catalases, and peroxidases. Superoxide dismutases (SODs) are ubiquitous metal-dependent enzymes that contain Cu and Zn, Mn, or Fe metal ions. All forms of SOD catalyze the dismutation of superoxide radicals into hydrogen peroxide and oxygen:

$$2\,{}^{\cdot}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

In the next stage of oxygen reduction, hydrogen peroxide is converted to water and oxygen by catalase:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

or peroxidase:

$$R_1SH + R_2SH + H_2O_2 \rightarrow R_1SSR_2 + 2H_2O$$

 © 2004, Elsevier Inc. All Rights Reserved. 

Almost any low-molecular-weight substance that contains a reducing moiety can react with ROS. Such nonenzymatic radical scavengers are present in significant quantities in cells or concentrated at ROS target sites. These scavengers include lipophilic substances ($\alpha$-tocopherol, $\beta$-carotene) and hydrophilic molecules (ascorbic acid, uric acid, glucose, and glutathione) that prevent transition metals from producing hydroxyl radicals, thereby performing an antioxidant function. The low concentrations of free $Fe^{2+}$ in living cells is a consequence of the presence of proteins in the cell that bind and sequester iron (ferritin, transferrin) or oxidize it (ceruloplasmin).

## DNA Damage

DNA is an important target of oxidative damage. Both the sugar-phosphodiester backbone and the bases are subject to attack by ROS, producing single- and double-strand breaks, intra- and interstrand cross-links, abasic (AP) sites, cyclonucleotides, and a variety of modified bases. ROS also damage DNA by lipid peroxidation. All DNA bases are subject to direct oxidation; the principal lesions detected in cellular DNA are shown in Figure 1.

The most prevalent oxidized base in DNA, 8-oxoguanine (8-oxoG), is formed by oxidation of guanine at C8. Like all purine nucleotides substituted at C8, 8-oxo-2′-deoxyguanosine (8-oxodG) adopts the *syn* rather than *anti* conformation unless additional restraints, such as Watson–Crick pairing, are present. In the *anti* conformation, 8-oxodG forms a Watson–Crick pair with dC, whereas in the *syn* conformation, it forms a Hoogsteen pair with dA.

## Biological Effects

### Cytotoxicity and Mutagenesis

The major cytotoxic effects of ROS represent responses to DNA double-strand breaks and lesions that block DNA replication. Certain oxidized bases are mutagenic, e.g., 8-oxoG accommodates a miscoding base (A) during DNA synthesis. The proofreading activity of DNA polymerases fails to remove this base when it is incorporated opposite 8-oxodG. Thus, mutations at 8-oxoG are predominantly G → T transversions. Interestingly, DNA polymerases differ significantly with respect to nucleotide insertion opposite 8-oxoG. Thus, replicative polymerases (DNA polymerase $\alpha$, DNA polymerase $\delta$, DNA polymerase III) preferentially insert dAMP opposite the damaged base, while polymerases involved in DNA repair (DNA polymerase $\beta$, DNA polymerase I) mainly incorporate dCMP opposite the lesion. Deoxynucleotide triphosphates are also subject to damage by ROS, promoting base misincorporation, which may lead to mutational events.

### Carcinogenesis

Oxidative stress is an important factor in tumor initiation and promotion. Its relative impact can be appreciated by noting that the amount of oxidized bases in DNA is significantly higher than the amount of DNA adducts formed by environmental mutagens. It is clear that somatic mutations play a central role in carcinogenesis and oxidative DNA damage has been implicated as an initiating event in this process. The role of ROS in tumor development also may be attributed, in part, to

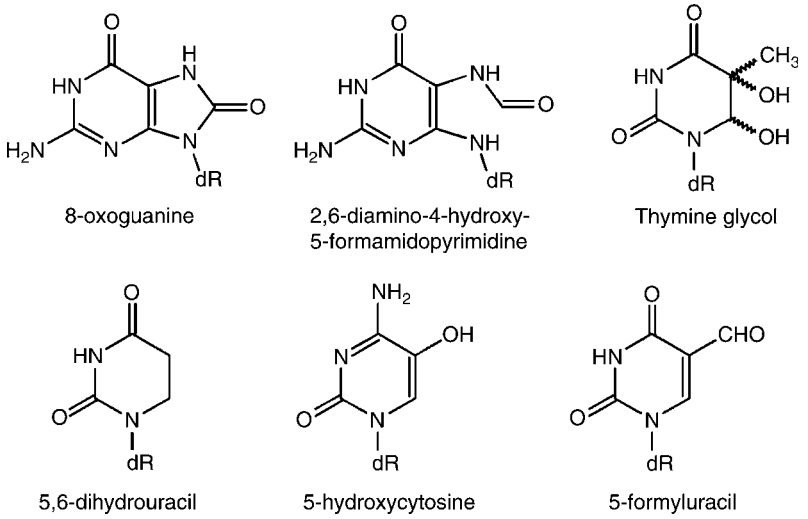



**FIGURE 1** Examples of major oxidative lesions in DNA.

their mitogenic properties and their ability to damage cellular proteins and membranes.

8-oxoG and thymine glycol are frequently used as biomarkers for oxidative DNA damage. Interestingly, tumor tissue sometimes contains increased amounts of oxidized bases. As most solid tumors are anoxic and generally display *lower* levels of general oxidative damage, this increase in DNA damage appears to be a cause rather than a consequence of neoplastic transformation. For tumor development to occur, a series of mutations are generally required to inactivate tumor suppressor genes and/or alter the status of protooncogenes. The presence of G → T transversions, characteristic of 8-oxoG, suggests that oxidative damage may have been involved in these instances. This signature base-substitution mutation is found in mutated p53 and *ras* genes in many human cancers and experimental systems.

### Aging

In 1956, D. Harman proposed a free-radical theory of aging. Oxidative DNA damage increases with age and the correlations between aging and DNA oxidation are reflected in increasing levels of 8-oxoG and thymine glycol. The relationship between an organism's life span and its ability to repair oxidatively damaged DNA has also been reported. Although the frequency of mutations increases with age, there are no documented cases of somatic mutations in the nuclear genome that are directly involved in causing age-related changes or age-related diseases (such as hypertension or Alzheimer's disease). There are, however, known, well-established mutations that are associated with certain spontaneous, nonhereditary cancers. Mitochondrial DNA (mtDNA), rather than nuclear DNA, has been proposed as a principal site of age-related damage accumulation. In fact, direct estimates of oxidative damage in mtDNA usually are higher than those in nuclear DNA. A specific, age-related deletion in the mtDNA of postmitotic cells, as well as multiple rearrangements, has been documented.

## Repair of Oxidative DNA Damage

### General

DNA damage occurs to a significant degree despite the ubiquitous presence of ROS scavengers. However, several cellular mechanisms are available to effect repair: direct reversal, base excision repair (BER), nucleotide excision (NER), methyl-directed mismatch repair, and recombination repair. Double-strand breaks are generally repaired by recombination repair; interstrand cross-links by a combination of NER and recombination repair; and intrastrand cross-links and cyclonucleotides by NER. Most oxidative damage is dealt with by BER. Here, damaged bases are excised by specific DNA glycosylases, creating an apurinic/apyrimidinic (AP) site. If the glycosylase has AP lyase activity, it can immediately cleave 3′ to the AP site; otherwise, a separate AP endonuclease cleaves 5′ to this position. Deoxyribose phosphate lyase (dRPase) removes the sugar residue, creating a substrate for a DNA polymerase. After this polymerase fills the resulting gap, a DNA ligase restores the double-stranded DNA.

Despite the wide variety of oxidatively damaged bases found in DNA, only a few glycosylases are involved in their repair. These enzymes necessarily exhibit broad substrate specificity, with some acting primarily on oxidized pyrimidines and others on oxidized purines. In *Escherichia coli*, oxidative damage to pyrimidines is repaired by endonucleases III and VIII (Nth and Nei); structurally related enzymes (NTH and NEIL) perform similar functions in eukaryotes. About 20 types of oxidized bases are excised by these DNA glycosylases. Depending on the type of chemistry performed by DNA glycosylases, modified AP sites are generated and processed by AP endonucleases to generate a suitable end for the DNA polymerase.

### GO System

8-oxoG presents special problems for DNA repair. Since either dAMP or dCMP can be incorporated opposite 8-oxodG, the repair system for this lesion must convert both 8-oxodG:dC and 8-oxodG:dA mispairs into a dG:dC pair. At least two cycles of BER are required to repair 8-oxodG:dA. First, adenine, the normal base, is excised, a process followed by insertion of dCMP opposite the lesion. The resulting 8-oxodG:dC mispair is then repaired by excision of 8-oxoG and subsequent insertion of dGMP.

In *E. coli*, a system of three enzymes – Fpg (MutM), MutT, and MutY (GO system) – counters the deleterious effects of 8-oxoG (Figure 2). MutT protein, a nucleoside triphosphate hydrolase, cleanses the cellular nucleotide pool of 8-oxodGTP. Fpg is an 8-oxoguanine-DNA glycosylase/AP lyase that excises 8-oxoG from 8-oxodG:dC but not from 8-oxodG:dA. MutY protein is an adenine glycosylase specific for dA:8-oxodG and dA:dG pairs. If 8-oxoG DNA arises by spontaneous oxidation of guanine or by incorporation of 8-oxodGMP opposite dC, it is excised by Fpg. However, if dA:8-oxodG is present because of incorporation of 8-oxodGMP opposite dA, it becomes a substrate for MutY. DNA polymerase I, the principal repair polymerase in *E. coli*, preferentially inserts dCMP opposite 8-oxodG. If dAMP is again inserted, the cycle of MutY repair may repeat. If dCMP is inserted during postreplication repair, the 8-oxodG:dA mispair is converted

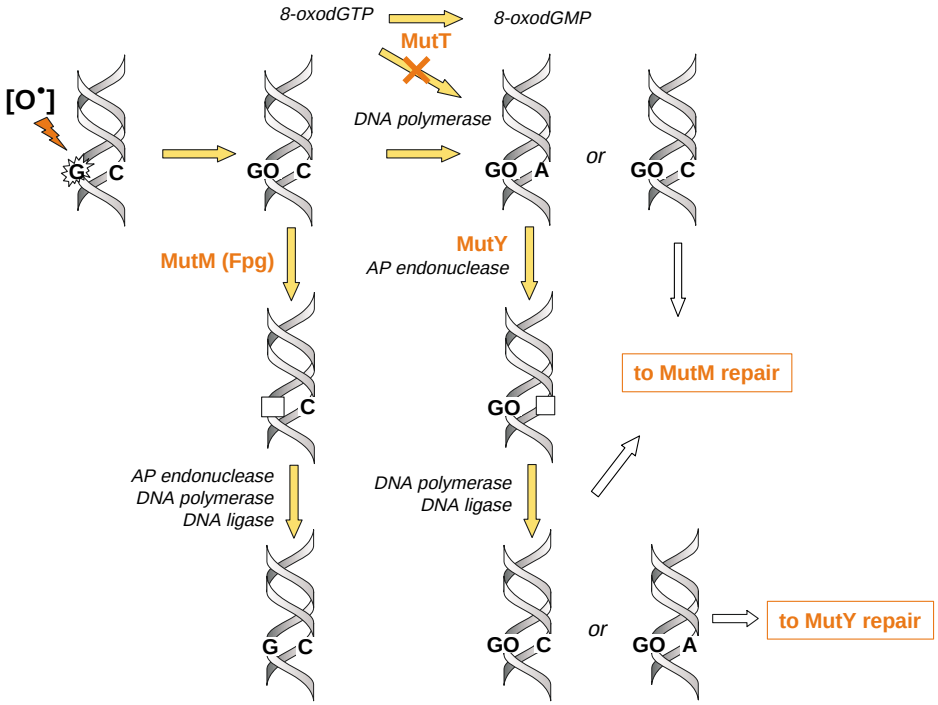



**FIGURE 2** GO system in bacteria.

to 8-oxodG:dC, a substrate for Fpg. This GO system also operates in most eukaryotes.

## See Also the Following Articles

DNA Glycosylases: Mechanisms • DNA Mismatch Repair and the DNA Damage Response • Superoxide Dismutase

## Glossary

**DNA damage** Any change in the bases (A, C, G, and T) or phosphodiester backbone structure that comprise canonical DNA.

**DNA repair** Any process, usually enzymatic, which participates in purging DNA of damaged elements and returns it to its canonical form.

**reactive oxygen species** A molecule that contains an oxygen atom with a highly reactive configuration of electrons.

## Further Reading

Aruoma, O. I., and Halliwell, B. (eds.) (1998). *DNA and Free Radicals: Techniques, Mechanisms, and Applications*. OICA International, London.

Friedberg, E. C., Walker, G. C., and Siede, W. (1995). *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.

Grollman, A. P., and Moriya, M. (1993). Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet.* **9**, 246–249.

Halliwell, B., and Gutteridge, J. (1999). *Free Radicals in Biology and Medicine*. Oxford University Press, Oxford.

Moldave, K., Mitra, S., and McCullough, A. (eds.) (2001). *Base Excision Repair (Progress in Nucleic Acid Research and Molecular Biology)*, *Vol 68*. Academic Press, London.

Stivers, J. T., and Jiang, Y. L. (2003). A mechanistic perspective on the chemistry of DNA repair glycosylases. *Chem. Rev.* **103**, 2729–2760.

von Sonntag, C. (1987). *The Chemical Basis of Radiation Biology*. Taylor and Francis, London.

## Biography

Arthur P. Grollman is Distinguished Professor of Pharmacological Sciences and Glick Professor of Experimental Medicine at the State University of New York at Stony Brook, where he heads the Laboratory of Chemical Biology. The Laboratory's research focuses on mechanisms of DNA damage, mutagenesis and DNA repair.

Dmitry O. Zharkov is a senior research scientist at the Novosibirsk Institute of Chemical Biology and Fundamental Medicine, Russia. His principal research interests are in the field of DNA repair and protein–nucleic acid interactions. He obtained a Ph.D. from the State University of New York at Stony Brook, where he later investigated the structure and mechanism of action of DNA glycosylases.

# DNA Photolyase

**Carrie L. Partch and Aziz Sancar**
*University of North Carolina School of Medicine, Chapel Hill, North Carolina, USA*

**Ultraviolet irradiation from sunlight damages DNA, and the growth of cells containing DNA lesions can lead to mutations, cell death, and cancer. Photolyase repairs UV-induced pyrimidine dimers by splitting the DNA photoproducts into individual pyrimidine bases through a process of photo-induced cyclic electron transfer using near-UV/blue light as an energy source. The reversal of the effect of far-UV (200–300 nm) by near-UV/visible light (300–500 nm), known as photoreactivation, is the most direct mechanism for repair of DNA photoproducts. Phylogenetically, the closest homolog of photolyase is a UV/blue light photo-receptor named cryptochrome, which regulates growth and development in plants and the circadian clock in animals and plants.**

## Classification

All members of the photolyase/cryptochrome family share a common evolutionary origin, high sequence homology, and identical chromophore/cofactors. Despite these similarities, their activities are directed toward unique substrates; photolyases repair either cyclobutane pyrimidine dimers or (6-4) photoproducts, induced in DNA by ultraviolet light. Cryptochromes do not have any repair activity; instead, they function as photoreceptors to regulate development in plants and synchronize circadian rhythms with the solar cycle in animals. Circadian rhythms are the daily oscillation of physiological processes designed to coordinate organismal activity with the solar cycle. Photoreactivation and circadian phototransduction may conceivably have had a common evolutionary origin. In the distant past, when more UV light reached the surface of the Earth, a primitive organism may have used the same protein to repair UV-induced DNA lesions and regulate daily behavior in order to minimize exposure to the harmful effects of sunlight.

### Species Distribution

The two members of the photolyase/cryptochrome family are found in many members of the three kingdoms of life. Cyclobutane photolyases are by far the most predominant across species. The two most abundant photoproducts formed by absorption of far-UV light (200–300 nm) by DNA are *cis,syn*-cyclobutane pyrimidine dimers (CPD) and pyrimidine–pyrimidone (6-4) photoproducts (Figure 1). UV induces cyclobutane pyrimidine dimers in DNA 10 times more frequently than (6-4) photoproducts. Many species that contain a cyclobutane photolyase lack a (6-4) photolyase, but those with a (6-4) photolyase always contain a cyclobutane photolyase, highlighting the importance of the cyclobutane photolyase for survival. Despite the relatively widespread distribution of photolyases in the three kingdoms, many microorganisms such as *Bacillus subtilis* and *Schizosaccharomyces pombe* do not have any photolyase. In contrast, *Escherichia coli* and *Saccharomyces cerevisiae* have cyclobutane photolyase and *Drosophila* has cyclobutane photolyase, (6-4) photolyase and cryptochrome. Of special interest, placental mammals such as mice and humans have two cryptochromes but do not possess either photolyase. UV photoproducts in these organisms are removed exclusively by the nucleotide excision repair pathway. Cryptochromes are expressed in plants, most animals, and some bacteria, frequently with more than one isoform in a species.

## Photolyase

### Primary Structure and Chromophores

Photolyases are 50- to 55-kDa monomeric proteins with two chromophore/cofactors. Sequence identity among the 50 or so known photolyases ranges from 15 to 70% with maximal sequence conservation in the catalytic FAD-binding domain. All photolyase/cryptochrome family proteins contain two noncovalently bound chromophores: a primary chromophore, FAD (flavin adenonucleotide), required for specificity in binding damaged DNA and catalysis, and a second, or "antenna" chromophore. The second chromophore is a folate (or rarely, a deazaflavin) and increases the rate of catalysis in

 © 2004, Elsevier Inc. All Rights Reserved. 

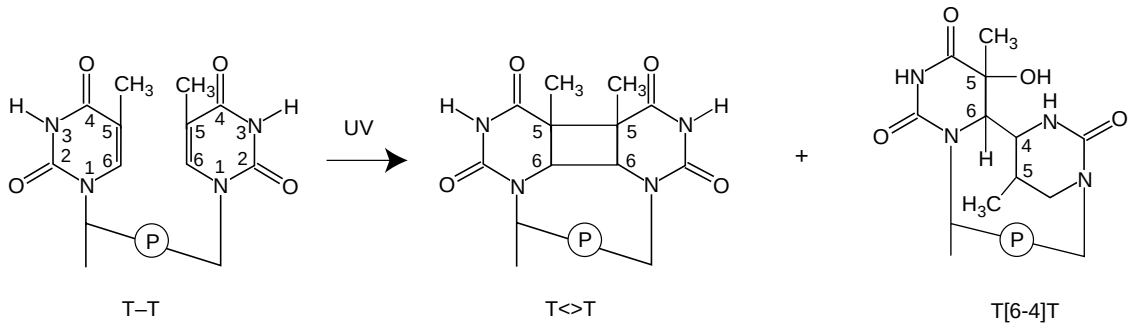



**FIGURE 1** Structure of UV-induced DNA photoproducts. The two major DNA photoproducts induced by far-UV irradiation are pyrimidine cyclobutane dimers (80–90% of lesions) and pyrimidine–pyrimidone (6-4) photoproducts (10–20% of lesions). The same type of photoproduct may form between any adjacent pyrimidines, including T–T, T–C, C–T, and C–C sites, except that (6-4) photoproducts do not form at C–T sites.

limiting light by absorbing light and transferring the energy to the FAD catalytic cofactor. Native photolyase contains FAD in the two-electron reduced and deprotonated form ($FADH^-$). This is the only active form of the cofactor. During purification the flavin is often converted to the catalytically inactive neutral radical ($FADH^\circ$) form, but it can be converted back to the $FADH^-$ either chemically or photochemically.

## Tertiary Structure

Crystal structures have been solved for several members of the photolyase family and despite their low sequence homology (~25% identity), the structures are remarkably similar. Photolyases exhibit a rather compact structure composed of two distinct domains: an N-terminal $\alpha/\beta$ domain (residues 1–131, *E. coli* photolyase numbering) and a C-terminal $\alpha$-helical domain (residues 204–471), connected to one another by a long loop (residues 132–203) (Figure 2). The FAD cofactor is buried deep within the $\alpha$-helical domain, held tightly in place by contact with 14 highly conserved amino acids. The antenna chromophore is bound loosely in a cleft between the two domains. An electrostatic representation of the surface of photolyase illustrates the presence of a large, shallow, positively charged groove that comprises the DNA-binding interface. A cavity in the middle of the groove allows the damaged nucleotide bases to fit in close proximity to the FAD during catalysis (Figure 2).

## Reaction Mechanism

The cyclobutane photolyase has been extensively studied and a detailed model for its reaction mechanism had been made. The enzyme binds pyrimidine dimers

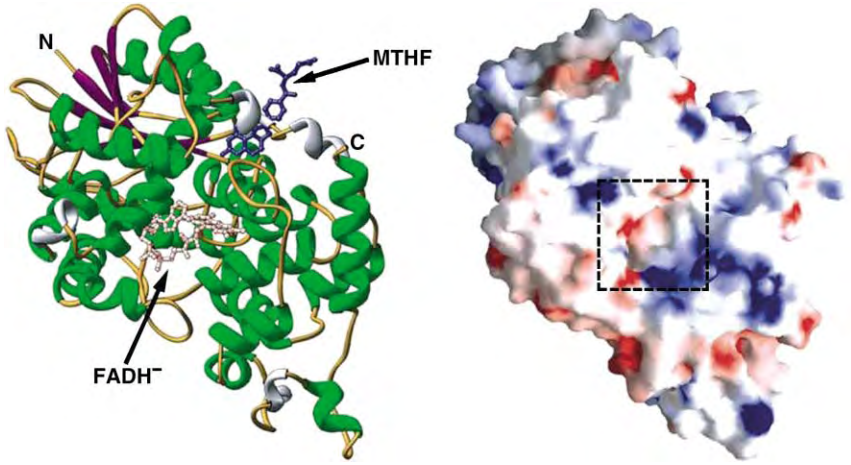



**FIGURE 2** The structure of *E. coli* photolyase. The ribbon diagram representation (left) of the crystal structure of *E. coli* photolyase obtained at 2.3Å shows the placement of the two chromophores and the distinct N-terminal $\alpha/\beta$ domain and C-terminal $\alpha$-helical domain. An electrostatic representation (right) of the surface illustrates the positive DNA-binding groove (shown in blue) that runs diagonally down the length of the protein and the dimer binding cavity (indicated by the dashed square).

independently of light, specifically attracted to the distorted DNA helix (bent approximately 30°) created by the DNA lesion. The dimer is flipped out of the double helix of the DNA into the active site cavity of photolyase, positioned in close proximity to the flavin (Figure 3A). Catalysis is initiated by light. A photon of near-UV/blue light (300–500 nm) is absorbed by the antenna chromophore and the excitation energy is transferred to the flavin ($FADH^-$) by fluorescence resonance energy transfer. The excited singlet state flavin $^1(FADH^-)^*$ then transfers an electron to the dimer, splitting the cyclobutane ring to form two pyrimidines. Concomitantly, an electron from the pyrimidine radical is transferred back to the neutral radical flavin ($FADH^\circ$) formed during catalysis to regenerate the active form of flavin ($FADH^-$) without a net redox change in the chromophore or substrate (Figure 3B). The repair of photoproducts by CPD photolyases occurs with a high quantum yield (photolesion repaired per absorbed photon) of 0.7–0.98.

The (6-4) photolyase has not been studied to the same extent as the cyclobutane photolyase, but it is believed to function in a similar manner with one significant difference. Upon binding the damaged DNA, the enzyme first thermally converts the (6-4) photoproduct to a four-membered ring intermediate closely resembling a cyclobutane dimer. The photo-induced electron transfer mechanism is presumed to be the same as for the classical cyclobutane photolyase, although there is no direct evidence for this mechanism and the quantum yield of repair is significantly lower for (6-4) photolyases, in the range of 0.05–0.10.

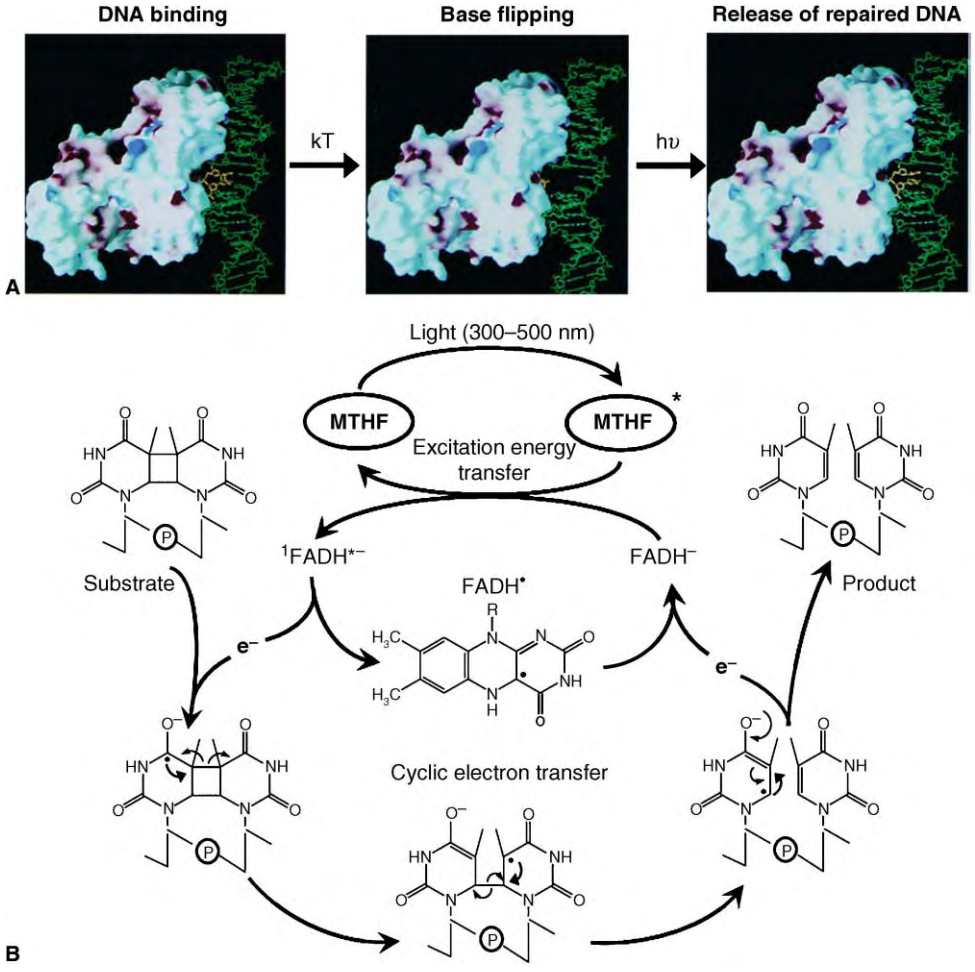



**FIGURE 3** Reaction mechanism of photoreactivation by *E. coli* photolyase. (A) General outline of the repair reaction. Photolyase binds to the damaged DNA (shown in yellow), flips the dimer into its active site cavity thermally (kT) for repair, and, following photorepair (h$\nu$), ejects the repaired DNA bases into the DNA helix. (B) Photochemical steps of dimer splitting. After absorption of a photon by the folate, energy is transferred to the flavin, which donates an electron to break the cyclobutane ring. An electron is transferred back from the product to the flavin, restoring the flavin to its ground state with no net change in redox status in either the substrate or cofactor.

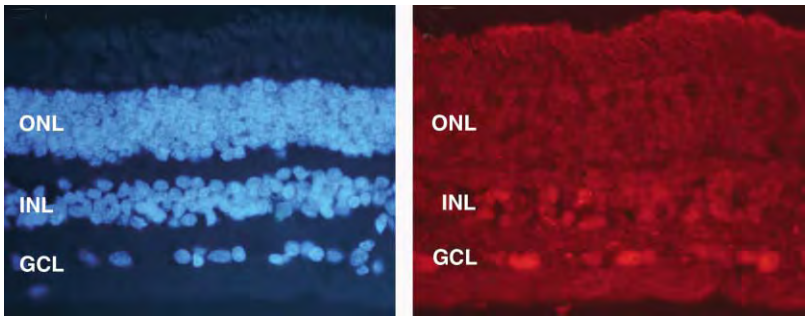



**FIGURE 4** Cryptochrome expression in the human retina. Human cryptochrome 2 (Cry2) is expressed in the inner retina. DAPI staining of nuclei (left, shown in blue) and Cry2 immunofluorescence (right, shown in red) are shown. Note the exclusive expression of Cry2 in the inner retina (INL and GCL). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

### Dark Repair Function

Even in the absence of photoreactivating light, photolyase plays an advantageous role in the cell by stimulating the repair of damaged DNA. This "dark function" is due to the stimulation of the nucleotide excision repair pathway by photolyase. As a general rule, the more a lesion distorts the DNA structure, the more efficiently it is recognized and removed by the excision nuclease system. Cyclobutane pyrimidine dimers cause only modest distortions of the DNA helix compared to (6-4) photoproducts and are repaired at a much lower rate in the absence of photolyase. Photolyase, presumably by binding the damage and flipping the dimer out of the helix, increases distortion of the DNA and accelerates the rate-limiting damage recognition step of the excision repair complex. In addition, photolyase recognizes DNA lesions caused by other chemical agents such as cisplatin, stimulating excision of the DNA adduct and increasing cellular survival without photoreactivation.

## Cryptochromes

### Structure

The basic architecture of cryptochrome is remarkably similar to photolyase. However, cryptochromes are structurally distinguished from photolyases by the presence of extended C-terminal domains ranging from 40 to 250 amino acids in length. These domains are thought to be important to the unique function of cryptochromes in circadian rhythms and growth control.

### Function

In nearly all organisms, the daily oscillation of physiological and behavioral processes such as sleep, body temperature, and metabolic rate are regulated by a transcription-based endogenous clock called the circadian (circa = about, dies = day) clock. These oscillations are kept in harmony with the solar cycle by photoreceptors that adjust the phase of the endogenous clock to coordinate rest or activity with the appropriate light cycle. Cryptochromes have two roles in maintaining circadian rhythms: photoreception and light-independent control of the endogenous clock. In mammals, circadian phototransduction is mediated solely through the eye and is not dependent on the outer retina containing the visual photoreceptors. Human cryptochromes are highly expressed in the inner retina (Figure 4). Elimination of cryptochromes by genetic means seriously compromises circadian phototransduction in mice and *Drosophila*, indicating that cryptochromes are the primary circadian photoreceptors. However, in the absence of cryptochromes there is some residual circadian photoreception, indicating that functional redundancy exists between cryptochromes and opsins in circadian phototransduction.

### Reaction Mechanism

The mechanism of photoreception/phototransduction by cryptochromes is not known at this time. However, catalytic residues from photolyase are conserved in cryptochromes and the unique C-terminal domains interact with effector proteins in a light-dependent manner in several organisms. It has been hypothesized that an intra- or intermolecular electron transfer mechanism may regulate accessibility of the C-terminal domain to effector proteins in response to light, thereby providing a regulated mechanism of signal transduction. The light-independent function of cryptochromes is to downregulate the expression of several clock genes by acting as a negative transcription factor.

### See Also the Following Articles

Nucleotide Excision Repair, Bacterial: The UvrABCD System • Nucleotide Excision Repair in Eukaryotes

## Glossary

**cryptochrome** Flavoprotein photoreceptor with homology to DNA photolyase that regulates the circadian clock.

**DNA adduct** Complex formed when a chemical is covalently bound to a DNA base.

**photolyase** Flavoprotein photoreceptor that uses UV/blue light to repair UV-induced photoproducts in DNA.

**pyrimidine dimer** Covalent linkage formed between two adjacent pyrimidine bases when DNA is exposed to far-UV light (200–300 nm).

## Further Reading

Carell, T., Burgdorf, L. T., Kundu, L. M., and Cichon, M. (2001). The mechanism of action of DNA photolyases. *Curr. Opin. Chem. Biol.* **5**, 491–498.

Sancar, A. (2000). Cryptochrome: The second photoactive pigment in the eye and its role in circadian photoreception. *Ann. Rev. Biochem.* **69**, 31–67.

Sancar, A. (2003). Structure and function of DNA photolyase and cryptochrome blue light photoreceptors. *Chem. Rev.* **103**, 2203–2237.

Sancar, A., Thompson, C., Thresher, R. J., Araujo, F., Mo, J., Ozgur, S., Vagas, E., Dawut, L., and Selby, C. P. (2000). Photolyase/cryptochrome family blue-light photoreceptors use light energy to repair DNA or set the circadian clock. *Cold Spring Harb. Symp. Quant. Biol.* **65**, 157–171.

Sancar, G. B. (2000). Enzymatic photoreactivation: 50 years and counting. *Mut. Res. Fund. Molec. Mech. Mutagen.* **451**, 25–37.

## Biography

Carrie Partch is a graduate student in the laboratory of Dr. Aziz Sancar at the University of North Carolina School of Medicine, Chapel Hill. She received her B.S. in 1997 from the University of Washington. She is investigating vertebrate cryptochrome function for her graduate work.

Aziz Sancar is a Distinguished Professor of Biochemistry and Biophysics at the University of North Carolina School of Medicine, Chapel Hill. He holds an M.D. from Istanbul University School of Medicine and a Ph.D. in molecular biology from the University of Texas at Dallas. He carried out his postdoctoral work at Yale University. He works on mechanisms of DNA repair and DNA damage checkpoints, as well as DNA photolyase/cryptochrome and circadian photoreception.

# DNA Polymerase $\alpha$, Eukaryotic

**Teresa S.-F. Wang**
*Stanford University School of Medicine, Stanford, California, USA*

**DNA polymerase $\alpha$ (pol$\alpha$) is a replicative DNA polymerase in eukaryotic cells essential for initiation of chromosome replication. Pol$\alpha$ exists in cells as a four-subunit enzyme complex often referred as pol$\alpha$-primase complex. The enzyme complex uniquely contains a RNA polymerase activity called primase activity that synthesizes a short RNA primer to sever as primer for the DNA polymerase activity of pol$\alpha$ to extend for DNA synthesis. This unique nature of pol$\alpha$-primase complex to perform *de novo* synthesis bequeaths it a critical role not only in initiation of chromosome replication but also in repair, recombination, checkpoint activation, telomere maintenance, and mutation avoidance. Thus, pol$\alpha$-primase plays a critical role in maintenance of genomic stability of cells.**

## Protein Structure and Subunit Components

DNA pol$\alpha$ consists of four subunits: a catalytic subunit of 165–180 kDa that contains the polymerase activity, a subunit of 70 kDa that has no detectable enzymatic activity but is essential for the initiation of replication both *in vivo* and *in vitro*, and two subunits with approximate molecular masses of 48 and 59 kDa that together contain the DNA primase activity. The ability of the primase subunits to synthesize a short RNA primer *de novo* renders pol$\alpha$ unique among other eukaryotic replicative DNA polymerases. The subunit composition of pol$\alpha$ from different organisms is conserved; their nomenclatures are listed (Table 1).

Although the predicted molecular mass of the pol$\alpha$ catalytic subunit calculated from the mammalian cDNA or yeast gene sequence is 165 kDa, the polypeptide often appears as a 180 kDa protein due to posttranslational modifications, including glycosylation and cell-cycle-dependent phosphorylation. A degraded form with a molecular mass of 160 kDa is also frequently seen. The B-subunit (p70) of human pol$\alpha$ as well as the budding yeast Pol12p protein are also phosphorylated in a cell cycle-dependent manner, whereas primase subunits from either human or yeast are not phosphorylated.

The human pol$\alpha$ catalytic subunit is localized on the short (p) arm of X chromosome at Xp21.3–22.1 (not on the q-arm as erroneously indicated in other review articles). The budding yeast *POL1* is localized on chromosome XIV and the fission yeast *pol$\alpha^+$* is localized on chromosome 1.

## Enzymatic Properties and Catalytic Mechanisms

### Optimal Conditions for DNA Polymerase Activity and Mechanisms of DNA Polymerase Action

Optimal assay conditions for DNA polymerase $\alpha$ activity include a pH of 8.0, a DNA primer-template with a gap size of 60–150 nucleotides, four deoxynucleotide triphosphates (dNTPs), and $Mg^{2+}$ as the metal ion activator. Pol$\alpha$ is sensitive to inhibitors such as aphidicolin and N-ethylmaleimide, and is highly sensitive to butylphenyl-dGTP and butylphenyl-dATP. In contrast to pol$\beta$ and pol$\gamma$, but similar to pol$\delta$ and pol$\varepsilon$, pol$\alpha$ is not sensitive to dideoxynucleotide triphosphates (ddNTPs).

Under the optimal assay conditions, pol$\alpha$ polymerizes DNA with moderate processivity and medium levels of fidelity, interacting first with template and then with primer. The minimum effective length of primer is eight nucleotides; the terminal three to five nucleotides of primer must be complementary to the template. Pol$\alpha$ can interact with a primer terminating in either 3′-H or 3′-OH, but not in 3′-$PO_4$. Following the interaction with template and primer, pol$\alpha$ recruits specific dNTPs as dictated by template nucleotide sequence. Hence, pol$\alpha$ interacts with its substrates in an ordered sequential mechanism.

### DNA Primase Activity and Mechanisms of Primer Synthesis

*De novo* initiation of primer synthesis requires the formation of a dinucleotide with a purine ribonucleotide triphosphate at the 5′ end by both primase subunits. Once the initial dinucleotide is formed, the catalytic

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE 1**

**Nomenclatures of Polα Subunits in Different Organisms**

| | Mammalian cells | Budding yeast (*Saccharomyces cerevisiae*) | | Fission yeast (*Schizosaccharomyces pombe*) | |
|---|---|---|---|---|---|
| | Protein | Protein | Gene | Protein | Gene |
| *Polymerase* | | | | | |
| Catalytic subunit | p180–p165 | Pollp | *POL1* | Polα | *polα*$^+$ |
| B-subunit | p70 | Poll2p | *POL12* | Spb70 | *spb70*$^+$ |
| *Primase* | | | | | |
| Catalytic subunit | p49 | Pri1p | *PRI1* | Spp1 | *spp1*$^+$ |
| Coupling subunit | p58 | Pri2p | *PRI2* | Spp2 | *spp2*$^+$ |

subunit of primase, p49, is sufficient to extend the ribo-primer. The formation of the initial dinucleotide suggests that the primase complex contains two proximate separate ribonucleotide triphosphate (rNTP)-binding sites. One site specifically binds the purine ribonucleotide triphosphate, while the other site offers the 3′-OH group of the ribonucleotide triphosphate to the second incoming ribonucleotide, located in the RNA polymerase domain of the heterodimeric primase protein complex. Although p49 is the catalytic subunit of primase, the p58 subunit is absolutely required for the p49 subunit to bind the purine ribonucleotide triphosphate to form the initial dinucleotide, and both the rate of extension of the ribo-primer and the binding affinity ($K_m$) of p49 for ribonucleotides (rNTPs) are influenced by the presence of p58 subunit. Immunoprecipitation analyses of the two primase subunits in cell extracts from both human cells and fission yeast cells indicate that the human p58 and fission yeast Spp2 physically couple the polymerase catalytic subunit p180 with the primase catalytic subunit, p49.

## Structure and Function of the Polymerase Catalytic Subunit

DNA polymerases are classified into A, B, C, X, and Y families, based on the similarities of their primary sequences to the three *E. coli* DNA polymerases I, II, and III, and to DNA polymerases from various organisms and DNA viruses. The primary sequences of polα from mammalian cells, budding yeast, and fission yeast cells contain six motifs that are highly conserved among many other prokaryotic, eukaryotic, and viral DNA polymerases. These conserved motifs are designated regions I–VI according to their extent of conservation; region I is the most conserved and region VI is the least conserved. Polymerases containing these six motifs are members of the B-family, or polα-family, of polymerases. Structural analysis of a polα-family member from bacteriophage RB69 (gp43) has revealed that the protein is shaped like a disk with a hole in the center that contains the active site. Similar to the Klenow fragment of *E. coli* Pol1, it also has a "right hand"-like structure composed of palm, finger and thumb domains. A 2.6 Å structural analysis of the ternary complex of RB69 polymerase in the presence of dTTP and duplex DNA primer-template revealed potential functions for many residues that have been confirmed by mutagenesis studies.

### Residues Responsible for Interactions with the Metal Ion-dNTP Complex

Analyses of the ternary complex of RB69 polymerase revealed two highly conserved motifs responsible for metal ion-dNTP binding in the polα-family (B-family) polymerases: -YGDTDS- and -DFNSLYPSII-, corresponding to regions I and II, also termed motifs C and A, respectively. These two motifs form a three-stranded anti-parallel β-sheet. The aspartate residue in region II (motif A) and the second aspartate residue in region I (motif C), together with the dTTP tail, coordinate two divalent metal ions for catalysis. Mutagenesis of these residues followed by steady-state kinetic analyses has confirmed that the second aspartate residue in region I (motif C) chelates the metal ion activator-nucleotide complex and is critical for catalysis. Structure analysis also indicates that the phenyl ring of Tyr (-Y-) in region II (motif A) stacks with the ribose ring of the incoming dNTP. This interaction is confirmed by mutagenesis studies in other polα-family polymerases. Furthermore, three positively charged residues in the finger domain: Arg (-R-) and a Lys (-K-) in region VI, interact with the γ-phosphate of dNTP and a Lys (-K-) in region III forms a hydrogen bond with the bridging oxygen between α- and β-phosphates of the dNTP. Comparison of the structures of apo RB69 polymerase and its ternary

complex indicates that binding of the incoming dNTP induces a 60° rotation of the finger domain allowing the three highly positive residues, Arg and Lys in region VI and Lys in region III, to move closer to the active site.

### Residues Responsible for Interactions with the Primer-Template DNA

Structural analyses of RB69 polymerase in complex with B-form duplex DNA have revealed that DNA primer-template binds in a groove between the palm domain and the thumb. Because residues involved in these interactions are not conserved among the polα-family (B-family) polymerases, the interactions most likely occur via hydrogen bonding between phosphates of the primer-template strand with the side chains of non-specific aggregates of residues. The polymerase protein or side chains directly form hydrogen bonds linking the primer-strand's phosphates, while a number of charged residues appear to interact with the template. The conserved motif of Lys-Lys-Arg-Tyr- (-KKRY-), unique to the polα-family polymerases, is involved in stabilizing the B-form DNA. The Tyr (-Y-) residue in this motif forms a hydrogen bond with the phosphodiester at the 3′-terminus of the primer, while the Lys (-K-) and Arg (-R-) interact with template phosphate to bring the primer and template strand backbones near one another.

## Biological Role in the Cell

### Initiation of Chromosome Replication

DNA polα primase has been regarded as the principal DNA polymerase involved in the initiation of chromosomal DNA synthesis. Reconstituted replication of an SV40 origin-containing plasmid by purified replication factors confirms that the polα-primase complex is essential to initiate both the leading and lagging strand DNA synthesis in a coordinated manner. Initiation of cell-free SV40 DNA replication requires the physical interaction of the SV40 viral large T-antigen in the pre-initiation complex with the amino terminal region residues 195–313 of the catalytic subunit of human pol α. After the synthesis of the initiation DNA primer (iDNA) by polα-primase complex, polα-primase complex is replaced by the more processive polδ, which extends the iDNA primer and completes elongation synthesis with the help of replication factor C (RFC) and proliferating cell antigen (PCNA). The human papillomavirus type 11 (HPV-11) origin recognition and initiator protein E1 also interacts with both the polα catalytic subunit (p180) and the B-subunit (p70) and the interactions are critical for the initiation of HPV-11 DNA replication. In addition, species-specific replication of polyomavirus (Py) DNA *in vitro* is dependent on the mouse primase catalytic subunit p48.

Finally, yeast cells harboring a conditional mutation in polα or primase exhibit an initiation defective phenotype when grown under restrictive conditions. Thus, biochemical studies of viral replication and yeast genetic analyses all indicate that the polα-primase complex is the principal cellular DNA polymerase for the initiation of chromosome replication.

### DNA Repair and Recombination

Mutations in budding yeast (*S. cerevisiae*) polα (*POL1*) or primase (*PRI2*) compromise completion of double strand break (DSB)-induced gene conversion at the *MAT* locus. Mutation of a glycine residue ($Gly^{493}$) to glutamate (Glu) in an evolutionarily conserved amino terminal region of budding yeast *POL1* induces a hyper-recombination (*hpr*) phenotype; mutation of this same $Gly^{493}$ residue to arginine (Arg) induces a high mutation rate, plasmid loss, and chromosome loss in budding yeast cells. Finally, mutation in budding yeast *POL12* (B-subunit of yeast) enhances the levels of ribosomal-DNA Holliday junction formation during S phase.

In fission yeast, developmentally programmed cell-type switching requires a strand-specific imprinting event at the mating type locus (*mat1*). This process requires the gene product of $swi7^{+}$, which encodes polα. A mutation in fission yeast *polα* can induce a defect in mating, indicating that polα is essential for mating type switching. Together, these findings indicate that polα is required for DNA repair and recombination processes in both mitotic and meiotic cells.

### Mutation Avoidance

Mutations in replication genes can often induce a mutator phenotype characterized by point mutations, single base frameshifts, and deletion or duplication of sequences flanked by short homologous repeats. Mutations in budding yeast polα (*pol1*) have been shown to induce dinucleotide repeat instability, deletion mutations, gross chromosomal alterations, and high frequencies of plasmid and chromosome loss. Among all of the replication mutators analyzed, mutations in the polα catalytic subunit (*pol1* or *polα*) in either budding or fission yeast induce a higher mutation rate in cells than do mutations in primase, polδ, polδ-subunits, and DNA ligase, indicating that polα plays an important role in mutation avoidance and genomic stability of cells.

### Checkpoint Activation

Checkpoints are surveillance mechanisms that monitor genomic integrity to signal the cell cycle machinery

monitor genomic integrity, the cell cycle machinery to delay mitosis to allow time for recovery and repair of replication perturbation or DNA damage. Genetic analyses and physiological studies of budding and fission yeasts have shown that checkpoint pathways are directly linked to DNA metabolism. Importantly, the establishment of a DNA replication fork is required for activation of checkpoint response. Since pol$\alpha$ and primase are essential for replication fork establishment, their functions are required for checkpoint activation to delay the mitotic entry until completion of DNA replication.

Genetic analyses of the budding yeast *pri1* mutant have suggested that the catalytic subunit of DNA primase (Pri1p) is linked to checkpoint response. In addition, genetic and biochemical studies of fission yeast primase (Spp1 and Spp2) have shown that Spp2 plays an essential role in maintaining the physical stability of pol$\alpha$-primase complex in cells, which has critical impact on the intra-S phase checkpoint kinase Cds1 (the homologue of budding yeast Rad53p and mammalian tumor suppressor Chk2). Fission yeast genetic data also indicate that the initiator DNA (iDNA) synthesized by pol$\alpha$-catalytic activity activates the checkpoint responsible for delaying premature mitotic entry during replication. Biochemical studies of *Xenopus* extracts, however, have shown that synthesis of RNA primer by primase is sufficient to activate the checkpoint.

Initiation defects caused by mutations in pol$\alpha$-primase can induce replication stalling and checkpoint activation. Checkpoint activation caused by mutation in a fission yeast pol$\alpha$ has been shown to promote tolerance through induction of mutagenic synthesis by error-prone translesional DNA polymerases $\kappa$ and $\zeta$. Furthermore, replication perturbation caused by mutations in the fission yeast pol$\alpha$ catalytic subunit activates the checkpoint kinase Cds1, which prevents mutagenesis and stabilizes the replication fork. Thus, pol$\alpha$-primase complex plays a direct as well as indirect role in checkpoint processes.

## Telomerase Stability and Telomere Length Maintenance

Eukaryotic chromosome ends are capped by telomeres consisting of simple tandem arrays of short G-rich repeats. Because DNA polymerases synthesize DNA in $5'-3'$ polarity, the telomeric G-rich strand is synthesized by the leading strand replication machinery and the C-rich strand is synthesized by the lagging strand replication machinery. Removal of the terminal RNA primer leaves a gap of 8–12 nucleotides at the $5'$-end of the newly synthesized lagging strand that cannot be synthesized by conventional DNA replication, resulting in a so-called "end replication problem". A special telomere synthesis polymerase, telomerase, containing an integral RNA molecule with a small template domain can add telomeric repeats onto the $3'$-end of the telomeric G-strand to resolve the end replication dilemma.

Studies of budding yeast have shown the pol$\alpha$-catalytic subunit physically interacts with the telomere binding protein Cdc13. Mutations in the budding yeast or fission yeast pol$\alpha$ catalytic subunit, as well as the fission yeast primase subunits Spp1 and Spp2, result in abnormal telomeric length extension. Moreover, the fission yeast pol$\alpha$ catalytic subunit physically associates with the telomerase catalytic subunit (Trt1). A mutation in fission yeast pol$\alpha$ that causes abnormal telomere lengthening also compromises both the pol$\alpha$-telomerase association and telomerase protein stability. Ectopic expression of wild type fission yeast pol$\alpha$ in this *pol$\alpha$* mutant can restore cellular telomerase protein to normal level and returns the telomere length to near wild type. Hence, the pol$\alpha$-primase complex co-exists with telomerase in the telomeric complex and is important for maintaining both telomere length equilibrium and telomerase protein stability.

## See Also the Following Articles

DNA Polymerase I, Bacterial • DNA Polymerase II, Bacterial • DNA Polymerase III, Bacterial • DNA Polymerases: Kinetics and Mechanism • DNA Polymerase $\beta$, Eukaryotic • DNA Polymerase $\delta$, Eukaryotic • DNA Polymerase $\varepsilon$, Eukaryotic • DNA Replication: Eukaryotic Origins and the Origin Recognition Complex • DNA Replication Fork, Eukaryotic • Translesion DNA Polymerases, Eukaryotic • umuC,D Lesion Bypass DNA Polymerase V • XPV DNA Polymerase and Ultraviolet Damage Bypass

## Glossary

**checkpoints** Signal transduction surveillance pathways that coordinate cell cycle transitions with the detection of unfinished DNA replication or damaged DNA to activate tolerance or repair processes to overcome the lesion or to induce apoptosis.

**fidelity of DNA polymerase** The error-free nature of a DNA polymerase in template-dictated incorporation of nucleotides.

**klenow fragment** A proteolytic fragment of *E. coli* Pol1 containing only the polymerase and the $3'-5'$ proofreading exonuclease domains.

**mutator** A gene mutation of which causes the cells to have additional new mutations.

**processivity of DNA polymerase** The uninterrupted repetitive DNA polymerase interaction with its DNA primer-template without dissociation.

## Further Reading

Bhaumik, D., and Wang, T. S.-F. (1998). Mutational effect of fission yeast polα on cell cycle event. *Molecul. Biol. Cell* **9**, 2107–2123.

Copeland, W. C., and Wang, T. S.-F. (1993). Enzymatic characterization of the individual mammalian primase subunits reveals a biphasic mechanism for initiation of DNA replication. *J. Biolog. Chem.* **268**, 26179–26189.

Dahlén, M., Sunnerhagen, P., and Wang, T. S.-F. (2003). Replication proteins influence the maintenance of telomere length and telomerase protein stability. *Molecul. Cell. Biol.* **23**, 3031–3042.

Franklin, M. C., Wang, J., and Steitz, T. A. (2001). Structure of the replicating complex of a polα family DNA polymerase. *Cell* **105**, 657–667.

Griffiths, D. J. F., Liu, V. F., Nurse, P., and Wang, T. S.-F. (2001). Role of fission yeast primase catalytic subunit in the replication checkpoint. *Molecul. Biol. Cell* **12**, 115–128.

Gutierrez, P. J. A., and Wang, T. S.-F. (2003). Genomic instability induced by mutations in *Saccharomyces cerevisiae POL1*. *Genetics* **165**, 65–81.

Kai, M., and Wang, T. S.-F. (2003). Checkpoint activation regulates mutagenic translesion synthesis. *Genes Develop.* **17**, 64–76.

Liu, V. F., Bhaumik, D., and Wang, T. S.-F. (1999). Mutator phenotype induced by aberrant replication. *Molecul. Cell. Biol.* **19**, 1126–1135.

Tan, S., and Wang, T. S.-F. (2000). Analysis of fission yeast primase defines the checkpoint responses to aberrant S phase initiation. *Molecul. Cell. Biol.* **20**, 7853–7866.

Wang, J., Sattar, A. K. M. A., Wang, C. C., Karam, J. D., Konigsberg, W. H., and Steitz, T. A. (1997). Crystal structure of polα family replication DNA polymerase from bacteriophage RB69. *Cell* **89**, 1087–1099.

## Biography

Dr. Teresa S.-F. Wang is a professor in the Department of Pathology at the Stanford University School of Medicine in Stanford, California. Her principal research interests are in enzymatic mechanisms of DNA polymerases and biological responses to mutations of DNA polymerases. She holds a Ph.D. from the University of Texas at Austin, Texas, and received her postdoctoral training at Stanford University.

# DNA Polymerase β, Eukaryotic

**William A. Beard and Samuel H. Wilson**
*National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA*

**DNA polymerase β (pol β) plays a crucial role in the base excision repair pathway that cleanses the genome of apurinic/apyrimidinic (AP) sites. AP sites arise in DNA from spontaneous base loss (depurination) and DNA damage-specific glycosylases that hydrolyze the *N*-glycosidic bond between the deoxyribose and damaged base. Pol β contributes two enzymatic activities, DNA synthesis and lyase, during the repair of AP sites. AP sites represent a potentially dangerous lesion to a cell since they can be mutagenic or cytotoxic.**

## Introduction

Endogenous and environmental agents continually threaten cellular DNA. These threats can result in physical damage (e.g., DNA strand breaks or base loss) or modification (e.g., alkylation and cross-links). Since these genetic insults lead to an altered DNA structure that can result in deleterious outcomes, cells have evolved DNA repair mechanisms to correct these abnormalities. The base excision repair (BER) pathway is responsible for removing simple base lesions and AP sites in DNA. The repair of an AP site minimally requires four coordinated enzymatic activities: strand incision by AP endonuclease, removal of the resulting deoxyribose phosphate (dRP) backbone of the AP site by a pol β-associated lyase, single-nucleotide DNA synthesis by pol β, and ligation of the DNA nick by DNA ligase. These steps are illustrated in Figure 1.

Pol β is the smallest eukaryotic cellular DNA polymerase (335 residues; 39 kDa) and it lacks a $3' \rightarrow 5'$ proofreading exonuclease activity that enhances the accuracies of replicative DNA polymerases (e.g., DNA polymerase ε and δ). Based on primary sequence alignments, pol β belongs to the X-family of DNA polymerases that also includes pol λ and pol μ.

## Biological Role

Based upon the high level of sequence conservation of pol β among mammalian species, it seemed highly likely that pol β is conducting a role that is essential for animal survival. This is consistent with the embryonic lethality of pol β null mice. Pol β preferentially fills short DNA gaps (<6 nucleotides). Because of this attribute, it had generally been assumed that pol β is involved in short-gap DNA repair synthesis. In addition, early studies implicated pol β in gap-filling DNA synthesis during mammalian BER of alkylation damage and the repair of UV DNA damage, and inhibitor studies also implicated pol β in other types of DNA repair, such as the repair of oxidative DNA damage. Although these attributes and studies implicated pol β in BER and in other types of short gap-filling DNA repair, pol β's role in BER was confirmed when mouse cells lacking pol β were found to be hypersensitive to DNA-damaging agents believed to be repaired by the BER pathway.

In addition to the "simple" single-nucleotide BER pathway illustrated in Figure 1, alternate BER pathways are needed to remove modified dRP-moieties that cannot be excised by the pol β lyase activity. In this situation, pol β strand displacement DNA synthesis creates a DNA flap of about four nucleotides with a 5′-modifed dRP group that can be subsequently removed by flap endonuclease-1 (FEN-1), thereby creating a nick that will be sealed by DNA ligase. This alternate pathway is referred to as "long-patch" BER.

## Pol β Domain Organization

Controlled proteolytic or chemical cleavage of pol β first demonstrated that it is folded into discrete domains. Subsequently, the X-ray crystal structure of the ternary substrate complex with DNA and an incoming nucleoside triphosphate (dNTP) defined the location of these domains in relation to the tertiary structure of the polymerase. Pol β is organized into two domains: an 8 kDa amino-terminal lyase domain and a 31 kDa carboxyl-terminal polymerase domain (Figure 2).

The structures of DNA polymerases derived from other polymerase families indicate that they also have a modular domain organization. The polymerase domain is typically composed of three functionally distinct subdomains. The catalytic subdomain coordinates two divalent metal cations that promote DNA synthesis. The other two subdomains are spatially situated on opposite

 © 2004, Elsevier Inc. All Rights Reserved. 

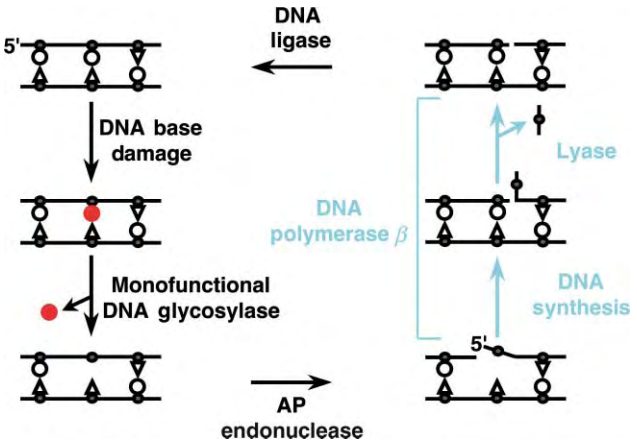



**FIGURE 1** Single-nucleotide BER. AP sites are lesions in DNA that originate from spontaneous or enzymatic (glycosylase) removal of a damaged base (red). These sites are removed from DNA by four coordinated enzymatic activities, two of which are contributed by pol β (blue).

sides of the catalytic subdomain. DNA polymerases outside the X-family of polymerases share structural homology within their catalytic subdomains. This has perpetuated a nomenclature based on an architectural analogy to a right hand where the subdomains are referred to as palm, thumb, and fingers. However, the catalytic subdomain of X-family members is not structurally homologous to other DNA polymerases. Application of the "hand analogy" to the polymerase subdomains of pol β is ambiguous. To highlight the intrinsic function of the polymerase subdomains of pol β, a functionally based nomenclature has been proposed. Accordingly, the pol β polymerase domain is composed of the C- (*C*atalytic), D- (*D*uplex DNA binding), and N-subdomains (*N*ascent base pair binding) (Figures 2 and 3). These correspond to the palm, thumb, and fingers subdomains of the right-handed DNA polymerases.

## Lyase Domain

The amino-terminal lyase domain (residues 1–90) has an associated lyase, metal-independent, activity that removes the 5′-dRP moiety generated during single-nucleotide BER (Figure 1). This activity plays a pivotal role in BER in that it represents the slowest step in the overall process, and if the lyase reaction is inhibited (e.g., modified dRP-moiety), alternate BER pathways will be initiated. The lyase reaction proceeds by β-elimination through a Schiff base intermediate. A stable covalently bound intermediate can be formed between the dRP-containing DNA substrate and the enzyme by $NaBH_4$ trapping. Lys72 is preferentially modified by primary amine reactive agents in both full-length pol β and the isolated lyase domain. Site-directed mutagenesis of Lys72 diminishes dRP lyase activity nearly 2 orders of magnitude indicating that this residue is the likely nucleophile that forms the Schiff base intermediate. The lyase domain is highly basic (net charge = +10), and the positively charged surface potential predicts that it would interact with the DNA backbone.

In addition to providing a crucial enzymatic activity for single-nucleotide BER, the lyase domain possesses a 5′-phosphate binding site that facilitates targeting of pol β into the 5′-position in a DNA gap. Importantly, pol β binds tightly to the 5′-phosphate only when there is DNA adjacent to the 5′-phosphate. This can be double-stranded or single-stranded DNA since a 3′-primer terminus is not required for optimum binding. Accordingly, pol β is expected to bind to the 5′-phosphate in a DNA gap of any size. The observation that pol β can processively (i.e., insert several nucleotides before dissociating from the DNA substrate) fill short gaps

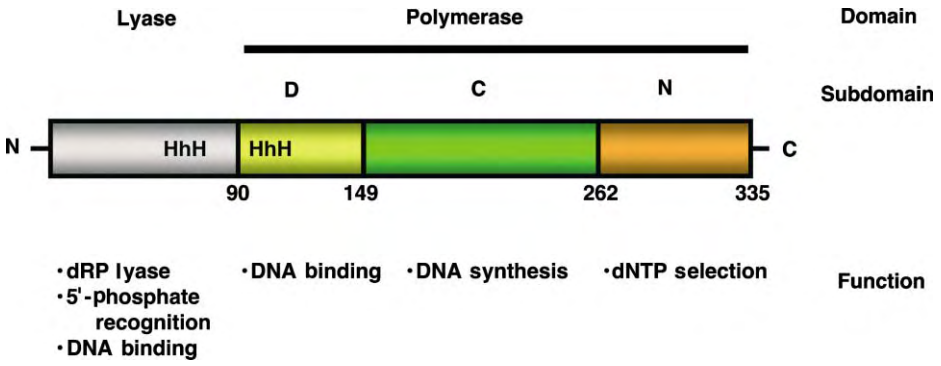



**FIGURE 2** Domain and subdomain organization of pol β. Pol β is composed of a polymerase (colored) and an amino-terminal lyase domain (gray). The polymerase domain is composed of three subdomains: D- (yellow), C- (green), and N- (orange) subdomains. These correspond to the thumb, palm, and fingers subdomains of DNA polymerases that utilize an architectural analogy to a right hand, respectively. The lyase domain and the D-subdomain each have a HhH motif that interacts with the DNA backbone of the incised DNA strand downstream and upstream of the gap, respectively.

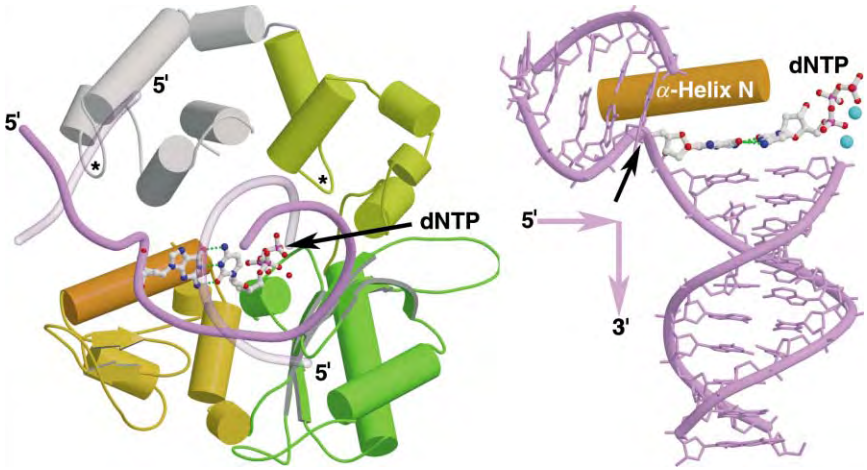



**FIGURE 3** Structure of the pol β-substrate complex. Left panel: The domains and subdomains are colored as in Figure 2 and the 5′-ends of the DNA strands forming a one-nucleotide gap are indicated. The trajectory of the template strand (purple) is altered dramatically as it enters the polymerase active site. The complementary DNA strands (primer and downstream oligonucleotides) are semitransparent. Except for the nascent base pair (templating and incoming nucleotides), the sugars/bases of the duplex DNA are not illustrated for clarity. An α-helix of the N-subdomain (dark orange) contributes one face of the binding pocket for the nascent base pair. The hairpins, of the HhH motifs, are highlighted with an asterisk. Right panel: The perspective is from the major groove edge of the nascent base pair and only α-helix N of pol β is shown for clarity. The 5′ → 3′ trajectory of the template strand is indicated and the radical bend in the 5′-phosphodiester bond of the templating base is highlighted (black arrow). The active site metals that coordinate the incoming dNTP and assist catalysis are illustrated as light blue spheres.

(<6 nucleotides) suggests that the lyase domain tethers the polymerase domain to the downstream position in gapped DNA. When the primer terminus (i.e., 3′-OH) is within six nucleotides of the 5′-phosphate on the downstream DNA strand, the polymerase domain will be "within reach" of the primer terminus thereby facilitating processive DNA synthesis.

The lyase domain is composed of five α-helices (Figure 3). Structural characterization of this domain has identified a structural motif that binds monovalent metals and interacts with the DNA backbone. The helix–hairpin–helix (HhH) motif (residues 55–79) has also been identified in other DNA repair proteins as well as many proteins that bind either single- or double-stranded DNA in a nonsequence-specific manner. The crystal structure of pol β bound to a one-nucleotide gap DNA substrate indicates that the lyase domain and the N-subdomain of the polymerase domain interact (Figure 3). The interaction of the lyase domain and the N-subdomain results in a donut-like structure. The interactions between these subdomains are altered upon binding substrates resulting in a tighter complex.

## Polymerase Domain

### D-Subdomain

As noted above, the polymerase domain is composed of three functionally distinct subdomains. The lyase domain is connected to the D-subdomain (residues 91–149) with a protease-hypersensitive hinge region. The D-subdomain interacts with the DNA sugar-phosphate backbone of the duplex DNA upstream of the polymerase active site. In addition to the HhH motif found in the lyase domain, a second HhH motif (residues 92–118) is also found in the D-subdomain. This HhH motif interacts with the primer strand phosphate backbone through a monovalent metal ion. Thus, the two HhH motifs in pol β are observed to make DNA backbone interactions with each end of the incised DNA strand. In the crystal structure of pol β bound with an incoming nucleotide and a one-nucleotide DNA gap, the DNA is bent ~90° (Figure 3). The DNA template does not travel through the hole in the donut-like structure. The sharp bend occurs at the 5′-phosphodiester bond of the templating base and is also observed in a product complex where pol β is bound to nicked DNA. The 3′-hydroxyl and 5′-phosphate groups on the nicked DNA strand, which need to be ligated in the final step of BER, are observed to be over 27Å apart. For pol β, the function of the HhH motifs is that a sequence nonspecific phosphate-backbone binding motif that stabilizes the pronounced bend observed in the pol β-gapped DNA structure. The 90° bend also exposes the terminal base pairs of each DNA duplex that is situated in the gap. His34 of the lyase domain interacts with the first base pair of the downstream duplex, whereas the N-subdomain contributes interactions with the nascent base pair (see below). The dramatic bend of the template strand as it enters the polymerase active site is a general feature observed in crystal structures of substrate complexes of all DNA polymerases.

### C-Subdomain

The C-subdomain (residues 150–262) of pol β includes three acidic residues that coordinate two essential magnesium ions. One magnesium coordinates nonbridging oxygens on the three phosphates of the incoming nucleotide (nucleotide-binding metal), whereas the other coordinates oxygens on the α-phosphate of the incoming dNTP and the DNA primer terminus (catalytic metal). These metals are believed to play critical and conserved roles in catalysis (nucleotidyl transfer) in all DNA polymerases. Although the C-subdomains of X-family members are not homologous to those of other polymerase families, crystal structures of substrate complexes of DNA polymerases from other families indicate that the reactive groups (i.e., metals, incoming nucleotide, and DNA) have a similar three-dimensional arrangement. Likewise, all DNA polymerases also follow an ordered binding of substrates where DNA binds first. The polymerase then selects a nucleoside triphosphate from a pool of structurally similar molecules to preserve Watson–Crick base pairing rules. The ability to choose between "right and wrong" is highly dependent on the identity of the polymerase. Pol β has a moderate ability to select the correct (i.e., right) nucleotide and capacity for DNA synthesis. Since it does not have an intrinsic proofreading activity, it has the potential to be highly mutagenic. The fidelity of pol β is too low (1 error per ~2000 nucleotides synthesized) for pol β DNA synthesis errors to be tolerated during DNA repair. It is generally believed that the proofreading activity of AP endonuclease, or some other extrinsic $3' \rightarrow 5'$-exonuclease, may proofread base substitution errors generated by pol β.

### N-Subdomain

The N-subdomain (residues 263–335) contributes important interactions to the binding pocket of the nascent base pair (templating and incoming nucleotides). Comparison of DNA polymerase structures bound to DNA with those that include an incoming complementary dNTP reveals that the N-subdomain repositions itself to "sandwich" the nascent base pair between the growing DNA terminus and the polymerase. In the absence of an incoming nucleotide, the N-subdomain is "open," but forms a "closed" complex upon binding a correct nucleotide. Thus, the dNTP-binding pocket is formed by the template base, DNA duplex terminus, and enzyme. For pol β, subdomain interactions with the nascent base pair are contributed primarily through α-helix N (Figure 3). These include stacking interactions with Lys280 and Asp276 with the templating and incoming nucleotide bases, respectively. Additionally, Asn279 and Arg283 contribute DNA minor groove interactions. Alanine substitution for Arg283 results in a dramatic decrease in fidelity. Since Arg283 plays a critical role in the formation of the closed complex, the low-fidelity mutant (i.e., R283A) is believed to be in an open conformation thereby losing the ability to promote efficient DNA synthesis when a correct nucleotide binds. Thus, the ability to choose the correct nucleotide has been lost. As noted above, the trajectory of the duplex DNA into the polymerase active site requires that the templating strand bend by 90° (Figure 3). This bend in the template strand serves at least two functions: (1) it provides access for the N-subdomain to check whether geometrical constraints imposed by correct Watson–Crick hydrogen bonding occurs; (2) it discourages the next templating base from entering the polymerase active site prematurely which could result in the incorrect template base coding for nucleotide insertion (deletion mutagenesis).

## Pol β–BER–Protein Interactions

In addition to its catalytic function, pol β also interacts with several proteins known to be involved in BER. These interactions may facilitate the coordination of the necessary enzymatic steps required in alternate BER pathways. In this context, the observations that pol β interacts with other DNA repair proteins such as AP endonuclease, DNA ligase I, X-ray cross-complementing factor-1 (XRCC1), poly(ADP-ribose) polymerase-1 (PARP-1), and proliferating cell nuclear antigen (PCNA), among others, suggest complex regulatory mechanisms that are not fully understood. Thus, pol β interacts with the enzymes involved in the steps immediately upstream and downstream of its own steps in single-nucleotide BER. Importantly, formation of binary or ternary protein complexes, rather than a "super" BER complex, has the advantage of flexibility that can accommodate alternate pathways. The influence of these protein–protein interactions on catalytic function and processing of BER intermediates remains to be determined.

## See Also the Following Articles

DNA Polymerase α, Eukaryotic • DNA Polymerase δ, Eukaryotic • DNA Polymerase ε, Eukaryotic • DNA Polymerases: Kinetics and Mechanism • Translesion DNA Polymerases, Eukaryotic• XPV DNA Polymerase and Ultraviolet Damage Bypass

## Glossary

**AP site** DNA lesion resulting from the loss of a base (i.e., abasic site). These sites can be mutagenic since they have lost their coding potential, or cytotoxic since they are quickly incised resulting in DNA strand breaks.

**DNA polymerase β** A small eukaryotic DNA polymerase involved in short gapped DNA repair synthesis. In addition to its polymerase activity, this polymerase contributes an accessory lyase activity required to remove the backbone of an AP site (i.e., deoxyribose) during BER.

**HhH** The helix–hairpin–helix structural motif binds single- or double-stranded DNA in a nonsequence-specific manner and has been identified in a number of DNA repair proteins. Two such motifs in pol β are observed to make DNA backbone interactions with the incised DNA strand downstream and upstream of the gap suggesting that they stabilize the pronounced DNA bend observed when gapped DNA binds to pol β.

**lyase** In the context of single-nucleotide BER, this reaction results in the removal of the 5′-deoxyribose phosphate backbone after incision of an AP site by an endonuclease. The reaction proceeds by β-elimination through a Schiff base intermediate. The ε-$NH_2$ group of a lysine side chain (Lys72 of pol β) serves as a nucleophile, resulting in transient covalent attachment of the enzyme to its substrate. This Schiff base intermediate can be trapped by sodium borohydride, resulting in conversion to an irreversibly linked lyase–DNA complex.

**N-subdomain** One of three subdomains found in all DNA polymerases. This subdomain forms one face of the nascent base pair (templating and incoming nucleotide bases) binding pocket and it is involved in selecting the correct dNTP. It is also referred to as the fingers or thumb subdomain in the polymerase nomenclature that utilizes the analogy to a right or left hand, respectively.

## Further Reading

Beard, W. A., and Wilson, S. H. (1995). Purification and domain-mapping of mammalian DNA polymerase β. *Methods Enzymol.* **262**, 98–107.

Beard, W. A., and Wilson, S. H. (2000). Structural design of a eukaryotic DNA repair polymerase: DNA polymerase β. *Mutat. Res.* **460**, 231–244.

Beard, W. A., and Wilson, S. H. (2003). Structural insights into the origins of DNA polymerase fidelity. *Structure* **11**, 489–496.

Beard, W. A., Shock, D. D., Vande Berg, B. J., and Wilson, S. H. (2002). Efficiency of correct nucleotide insertion governs DNA polymerase fidelity. *J. Biol. Chem.* **277**, 47393–47398.

Horton, J. K., Joyce-Gray, D. F., Pachkowski, B. F., Swenberg, J. A., and Wilson, S. H. (2003). Hypersensitivity of DNA polymerase β null mouse fibroblasts reflects accumulation of cytotoxic repair intermediates from site-specific alkyl DNA lesions. *DNA Repair (Amst.)* **2**, 27–48.

Lindahl, T., and Wood, R. D. (1999). Quality control by DNA repair. *Science* **286**, 1897–1905.

Sawaya, M. R., Prasad, P., Wilson, S. H., Kraut, J., and Pelletier, H. (1997). Crystal structures of human DNA polymerase β complexed with gapped and nicked DNA: Evidence for an induced fit mechanism. *Biochemistry* **36**, 11205–11215.

Sobol, R. W., Horton, J. K., Kühn, R., Gu, H., Singhal, R. K., Prasad, R., Rajewsky, K., and Wilson, S. H. (1996). Requirement of mammalian DNA polymerase β in base excision repair. *Nature* **379**, 183–186.

Wilson, S. H., and Kunkel, T. A. (2000). Passing the baton in base excision repair. *Nat. Struct. Biol.* **7**, 176–178.

## Biography

Samuel H. Wilson is Head of the DNA Repair and Nucleic Acid Enzymology Section in the Laboratory of Structural Biology at NIEHS/NIH in Research Triangle Park, NC. Prior to this, Dr. Wilson was the founding Director of the Sealy Center for Molecular Science at the University of Texas Medical Branch-Galveston.

William A. Beard holds a Ph.D. from Purdue University and did postdoctoral training at St. Jude Children's Research Hospital, Memphis. He joined Dr. Wilson's group in 1990 and is currently a Staff Scientist in the Laboratory of Structural Biology at NIEHS/NIH.

Their common research interests are to understand basic mechanisms of biological catalysis and substrate recognition through a combination of site-directed mutagenesis, kinetic analysis, molecular modeling and structure determination. They have effectively applied these approaches to derive a molecular and biological description of DNA polymerase β function.

# DNA Polymerase δ, Eukaryotic

**Antero G. So and Kathleen M. Downey**
*University of Miami, Miami, Florida, USA*

**DNA polymerase δ (pol δ) is an essential DNA polymerase that is required for DNA replication and participates in several DNA repair pathways. It is a proofreading DNA polymerase; that is, it is capable not only of catalyzing template-directed nucleotide incorporation, but also of editing errors of incorporation by virtue of a 3′ to 5′ exonuclease activity. Pol δ is highly conserved and has been identified in multiple eukaryotic species from yeast to humans.**

## Structure

Pol δ comprises a core enzyme and one or more loosely associated subunits.

### The Core Enzyme

The pol δ core enzyme is a heterodimer comprising a catalytic subunit of approximately 125 kDa and a small subunit of approximately 50 kDa. The catalytic subunit contains the active sites for both the DNA polymerase and 3′ to 5′ exonuclease activities, as well as a putative nuclear localization signal. The small subunit is not associated with any catalytic activity, but it is required for the interaction of the core enzyme with more loosely associated subunits and accessory proteins.

Cloning and sequencing of the cDNAs for the catalytic and small subunits of pol δ from various species demonstrated that pol δ is the most highly conserved among the eukaryotic DNA polymerases, with sequence identity of 94% between both subunits of the human and bovine enzymes and 44% identity between the subunits of the human and budding yeast enzymes. The human gene for the catalytic subunit of pol δ has been mapped to chromosome 19q13.3 and that for the small subunit has been located on chromosome 7.

Pol δ belongs to the class B, or α-like, DNA polymerases, which are found in both prokaryotes and eukaryotes. The class B polymerases include pol α, pol δ, pol ε, the herpes virus family of DNA polymerases, and the T4 and RB69 bacteriophage DNA polymerases. These enzymes contain six highly conserved domains and, in addition, pol δ also contains three highly conserved regions (ExoI, ExoII, and ExoIII), which are responsible for the 3′ to 5′ proofreading exonuclease activity and are found in all exonuclease-containing DNA polymerases.

Pol δ is very sensitive to inhibition by aphidicolin, N-ethylmaleimide, and carbonylphosphonate; moderately sensitive to butylphenyl-dGTP and butylphenyl-dATP; and resistant to dideoxynucleotides.

### Other Subunits

The third subunit of pol δ is a polypeptide of 42–66 kDa that has been found in both mammalian and yeast enzyme preparations. It interacts with both the small subunit of the enzyme and the processivity factor for pol δ, the proliferating cell nuclear antigen (PCNA). The fourth subunit of pol δ, which has been identified only in fission yeast and mammalian cells thus far, is a polypeptide of approximately 12 kDa, whose function has not yet been elucidated.

Several other proteins have been identified as interacting specifically with the small subunit of pol δ. In addition to the third subunit, these proteins include the Werner's syndrome protein (WRN) and pol δ interacting protein 1 (PDIP1). All of these proteins have been shown to physically and functionally interact with the small subunit of pol δ as well as with its processivity factor PCNA.

### Processivity Factor

Core pol δ is essentially a distributive DNA polymerase that is capable of synthesizing only short stretches of DNA (10–20 nt) before dissociating from the template. However, when bound to its processivity factor PCNA, pol δ becomes a highly processive enzyme, capable of synthesizing stretches of DNA containing up to $5 \times 10^4$ nucleotides before being released from the template. PCNA is a homotrimer that has a subunit size of approximately 29 kDa and forms a ring with an internal diameter capable of accommodating double-stranded DNA. PCNA, which has been called a sliding clamp, is

 © 2004, Elsevier Inc. All Rights Reserved. 

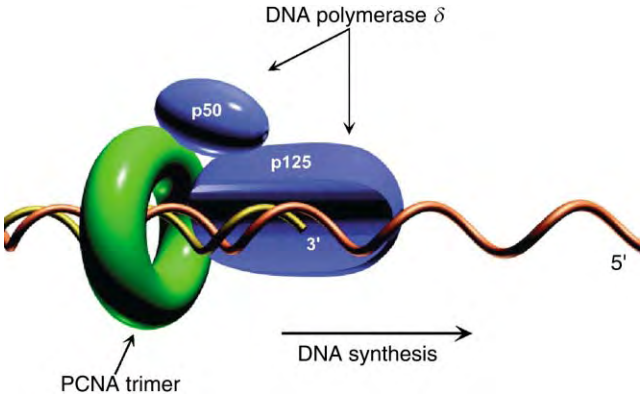



**FIGURE 1** Schematic model of the pol δ-PCNA–DNA complex during DNA replication. Reprinted from Mozzherin, D. J., Tan, C.-K., Downey, K. M., and Fisher, P. A. (1999) Architecture of the active DNA polymerase d–proliferating cell nuclear antigen–template primer complex. *J. Biol. Chem.* **274**, 19862–19867 with permission from the American Society for Biochemistry and Molecular Biology.

loaded onto DNA by a clamp loader, replication factor C (RFC), in a reaction that requires ATP hydrolysis. PCNA binds to pol δ and, by virtue of being topologically linked to the DNA template primer, causes pol δ to become highly processive (see Figure 1).

Functional studies with recombinant p125 from human, mouse, and fission yeast have shown that DNA synthesis catalyzed by p125 alone is not stimulated by PCNA, whereas the activity and processivity of the recombinant human p125/p50 heterodimer is fully stimulated by PCNA to the same extent as the native two-subunit enzyme isolated from calf thymus tissue, suggesting that p50 is required for the functional interaction of PCNA with pol δ.

## Functions of Pol δ

### DNA Replication

There is strong evidence from studies in both higher and lower eukaryotes that pol δ plays an essential role in DNA replication. The development of an *in vitro* simian virus 40 (SV40) DNA replication system led to the identification of both pol δ and PCNA as mammalian replication proteins. Furthermore, studies in both fission and budding yeast have shown that the catalytic and small subunits of pol δ are the products of essential genes, and the phenotypes of mutants that are temperature-sensitive in these genes are consistent with a role for pol δ in replication. However, the precise role of pol δ at the replication fork is still unclear. Although it is clear that pol δ is capable of catalyzing the synthesis of the leading strand as well as elongation of the lagging-strand Okazaki fragments, after priming of these strands by the action of pol α-primase, whether either of these functions is exclusively attributable to pol δ *in vivo* and whether pol ε can carry out these functions have not yet been resolved.

### DNA Repair

Although there are highly specialized DNA polymerases that are involved in the repair of specific lesions in DNA, the replicative polymerases pol δ and pol ε appear to carry out the bulk of DNA synthesis involved in filling in the gaps generated during the repair of DNA damaged by ultraviolet (UV) irradiation or the chemical alteration of bases (i.e., nucleotide excision repair and base excision repair). Genetic studies in yeast and biochemical studies in mammalian cells have also demonstrated a role for pol δ in postreplication mismatch repair.

## See Also the Following Articles

DNA Mismatch Repair in Mammals • DNA Polymerase α, Eukaryotic • DNA Polymerase β, Eukaryotic • DNA Polymerase ε, Eukaryotic • DNA Replication: Eukaryotic Origins and the Origin Recognition Complex • Translesion DNA Polymerases, Eukaryotic

## Glossary

**base excision repair** A repair characterized by excision of nucleic acid base residues in free form.

**nuclear localization signal** The amino acid sequence responsible for proteins being actively and selectively imported into the nucleus.

**nucleotide excision repair** A repair in which damaged nucleotides are removed as part of DNA fragments.

**processivity** The extent of nucleotide incorporation per DNA polymerase binding event.

**proofreading** The correction of mispaired nucleotides at the end of a growing DNA chain during replication.

## Further Reading

Burgers, P. M. J. (1998). Eukaryotic DNA polymerases in DNA replication and DNA repair. *Chromosoma* **107**, 218–227.

DePamphilis, M. L. (1996). *DNA Replication in Eukaryotic Cells.* Cold Spring Harbor Laboratory Press, New York.

Hubscher, U. (1997). DNA polymerase δ, an essential enzyme for DNA transactions. *Biol. Chem.* **378**, 345–362.

Kornberg, A., and Baker, T. (1992). *DNA Replication.* W. H. Freeman and Company, New York.

Waga, S., and Stillman, B. (1998). The DNA replication fork in eukaryotic cells. *Annu. Rev. Biochem.* **67**, 721–751.

## Biography

Antero G. So is a Professor in the Departments of Medicine and of Biochemistry and Molecular Biology at the University of Miami. His principal interest is in the field of mammalian DNA replication, with major emphasis on mammalian DNA polymerases. He holds an M.D. from the University of Santo Tomas and a Ph.D. from the University of Washington; he did his postdoctoral training at Case Western University and the University of Geneva.

Kathleen M. Downey is a Professor in the Departments of Medicine and of Biochemistry at the University of Miami. Her principal interest is in mammalian DNA replication and repair. She holds a Ph.D. from the University of Washington in Seattle, Washington and received her postdoctoral training at the Swiss Federal Institute of Technology in Zurich, Switzerland.

# DNA Polymerase ε, Eukaryotic

**Yasuo Kawasaki and Akio Sugino**
*Osaka University, Suita, Osaka, Japan*

**DNA polymerase ε is one of the least understood cellular DNA polymerases. Recent studies have revealed that it plays a central role in chromosomal DNA replication. DNA polymerase ε activity was reported in the extract from rabbit bone marrow cells in 1985. Although DNA polymerase ε was purified from calf thymus and human cells in the late 1980s, it was described initially as a "large form" of DNA polymerase δ or PCNA-independent DNA polymerase δ whose activity is not affected by proliferating cell nuclear antigen (PCNA). This DNA polymerase is a high-molecular-weight, aphidicolin-sensitive enzyme that copurifies with a tightly associated 3′–5′ exonuclease. In addition, it is highly processive in the absence of polymerase-loading factor, PCNA. In budding yeast *Saccharomyces cerevisiae*, DNA polymerase activity called DNA polymerase II was already known in the 1970s. The complete enzyme complex was purified in 1990 and it turned out to be the yeast homologue of DNA polymerase ε. It was in 1990 that the nomenclature of "DNA polymerase ε" was established. Since then, molecular structure and biological function have been extensively studied especially in budding yeast.**

## Molecular Structure

DNA polymerase ε (Pol ε) is purified from yeast as a four-subunit holoenzyme. It consists of four subunits, Pol2p, Dpb2p, Dpb3p, and Dpb4p in budding yeast and p261, p59, p12, and p17 in human. The genes encoding all four subunits of yeast and human enzyme have been cloned and sequenced. The genes for the largest and the second largest subunit, *POL2* and *DPB2* respectively, were shown to be essential for budding yeast cell growth, while *DPB3* and *DPB4* are not essential.

### Catalytic Subunit

The largest subunit of budding yeast Pol ε is encoded by *POL2* gene and the molecular mass is 256 kDa. Its human counterpart, encoded by *POLE*, is 261 kDa. Homologues in other eukaryotes were also found including that in fission yeast *Schizosaccharomyces pombe*, encoded by *cdc20*$^+$ gene. The catalytic domain has the consensus amino acid sequences common to B family DNA polymerases (Figure 1). Eukaryotic DNA polymerases α, δ, ε, and ζ, *Escherichia coli* DNA polymerase II, and several viral DNA polymerases (T4 phage, HSV, and EBV) belong to B family. Unlike the other catalytic subunits of eukaryotic B family DNA polymerases, Pol2p has a long stretch at carboxyl-terminal half that is not related to the catalytic activity (Figure 1). There are two putative zinc-finger motifs at the carboxyl terminus, which is required for complex formation with the other three subunits. Overlapping partly with the catalytic domain, it has 3′–5′ exonuclease activity, which is responsible for editing during DNA biosynthesis. These molecular structures are conserved in all eukaryotes.

### Other Subunits

The second largest subunit of Pol ε is encoded by the budding yeast *DPB2* gene, which is essential for viability, and by *POLE2* in humans. The molecular mass is 80 kDa in yeast and 59 kDa in humans, and they share 26% amino acid sequence identity. The function of this subunit is not known yet.

Budding yeast 34 kDa subunit of Pol ε is encoded by *DPB3* gene and the 29 kDa subunit is encoded by *DPB4* gene. These genes are not essential for growth since deletion mutants are viable. However, the DNA polymerase activity from either *DPB3* or *DPB4* deletion strain is less than that from wild-type cells, presumably because the complex formation is unstable. p17 and p12 correspond to the human homologues of Dpb4p and Dpb3p. They interact physically with p261 and p59, although a four-subunit human holoenzyme has not yet been isolated. These two subunits have a so-called histone-fold motif that is found in many transcription factors and is utilized for interaction with DNA. However, the significance of these motifs with respect to biological function of Pol ε is not yet known.

## Biochemical Character

The biochemical properties of Pol ε are very similar to those of DNA polymerase δ; its activity is sensitive to aphidicolin, resistant to deoxyribonucleotide analogue

 © 2004, Elsevier Inc. All Rights Reserved. 

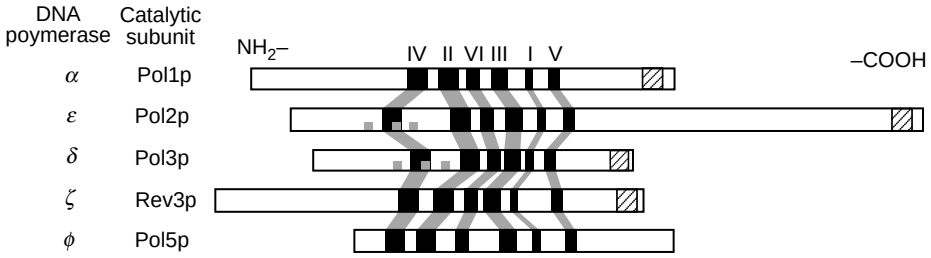



**FIGURE 1** Eukaryotic B family DNA polymerases. DNA polymerases that possess B family catalytic domain are aligned. Closed boxes (I–V) represent the subdomains conserved among B family DNA polymerases. Gray boxes represent the exonuclease domains. Shaded boxes represent cysteine-rich domain which can form Zinc-fingers.

ddTTP, and characterized by high fidelity. Pol ε is distinguished from DNA polymerase δ by its high processivity in the absence of the cofactors PCNA, RFC, and RPA. However, under some conditions, it complexes with and is stimulated by PCNA, although the physiological significance of this interaction is unknown. During purification of Pol ε from yeast or mammalian cells, a small form of Pol2p ("145-kDa form" in yeast) has been identified in addition to the holoenzyme (Figure 2). Purified 145-kDa form, which lacks the carboxyl-terminal half, does not associate with the other three subunits. Both 145-kDa form and holoenzyme are highly processive and have an intrinsic proofreading $3'-5'$ exonuclease activity.

## Role in DNA Replication

### Role at Replication Fork

The SV40 DNA replication system, which serves as a model for eukaryotic DNA replication, has been successfully reconstituted *in vitro* with SV40 large T-antigen and mammalian proteins. Analysis of this system, which includes DNA polymerases α and δ, led to the conclusion that DNA polymerase α and δ are sufficient for synthesis of both leading and lagging strands in eukaryotes. These studies showed that DNA polymerase α, which is associated with primase activity, is required for initiation of new DNA chains. Only DNA polymerase δ was required to replicate both leading and lagging strands, leaving a replication role for Pol ε in doubt. However, several lines of evidence indicate the presence of Pol ε at the eukaryotic replication fork. Evidence for a physical interaction came from cross-linking experiments, which showed that Pol2p is located at or near the replication fork. Furthermore, the mutation rate is increased about tenfold in a yeast strain carrying a *pol2* mutation that inactivates the Pol ε proofreading capability (mutation in $3'-5'$ exonuclease domain) suggesting that this enzyme contributes to the synthesis of newly replicated DNA. A yeast double mutant lacking proofreading

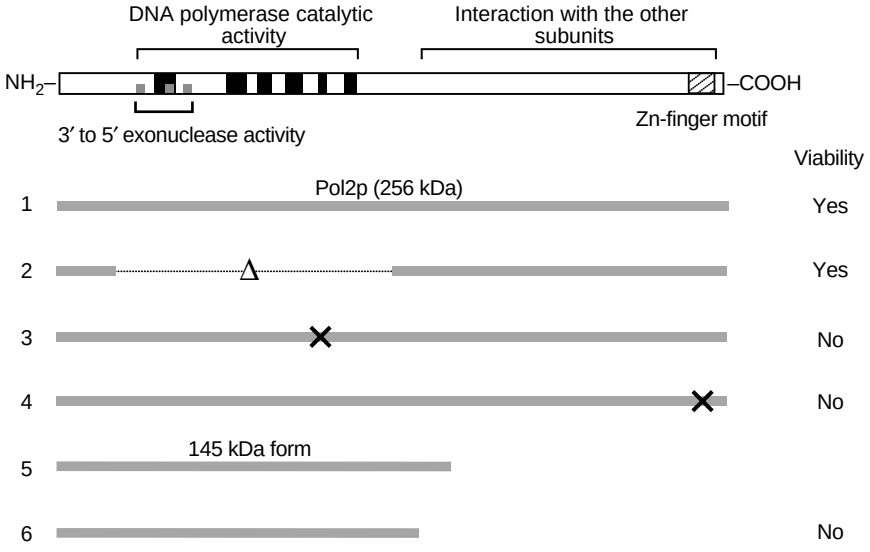



**FIGURE 2** Domain structure of the yeast Pol2p subunit. Closed, gray, and shaded boxed are as described in Figure 1. Mutated or truncated versions of Pol2p are schematically shown and the viability of yeast cells which express each mutant Pol2p are indicated. 1, full-length Pol2p; 2, complete deletion of catalytic domain; 3, missence mutation in domain I; 4, missence mutation in zinc-finger motif; 5, endogenous 145 kDa form; 6, deletion of carboxyl terminus.

capability of both Pol2p and Pol3p cannot survive, presumably because the mutations accumulate to a lethal level. In addition, it has also been shown that loss of the exonuclease activity in either Pol2p or Pol3p alters the mutation spectra in a strand-specific manner. It has also been shown that DNA replication activity was drastically lost in *Xenopus* egg extracts when Pol ε was removed immunologically from the egg extract. Several conditionally lethal mutants of *POL2* have been isolated and characterized in budding yeast. Chromosomal DNA synthesis in these mutant cells ceases under the restrictive condition, indicating that Pol ε is required for normal chromosomal DNA replication. Consistent with this conclusion, the terminal morphology of mutant cells is the same as those of other DNA replication mutants in budding yeast, namely a dumbbell shape with a single nucleus localized between mother and daughter cells. Furthermore, a temperature-sensitive mutant of the *DPB2* gene also results in temperature-sensitive chromosomal DNA replication. Pol ε also exhibits the highest processivity among replicative DNA polymerases, suggesting that Pol ε synthesizes the leading strand, while the lagging strand is synthesized by DNA polymerase α and δ in a discontinuous manner.

### Distinct Roles and Domain Structure

Yeast cells carrying a point mutation in the conserved catalytic domain of Pol2p are not viable. By contrast yeast mutant cells that have an internal deletion of *POL2*, resulting in loss of the DNA polymerase catalytic domain and the 3′–5′ exonuclease domain, can survive (Figure 2). However, this strain is temperature sensitive for cell growth, exhibits slow growth phenotype even at permissive temperature, and the elongation of DNA strands in the mutant cells is slow. This apparent discrepancy can be explained by considering the discrete functional domains of Pol ε. Although Pol ε may normally be present at the replication fork, in the complete absence of the catalytic domain, another DNA polymerase, presumably DNA polymerase δ, can substitute. On the other hand, the carboxyl terminus that is dispensable for the processive DNA synthesis *in vitro* is essential for growth (Figure 2). The *in vivo* requirement of carboxyl terminus may involve function in the initiation of DNA replication via interaction with the other subunits.

## Role in DNA Repair

Apart from DNA replication, Pol ε participates in DNA repair and recombination. It was first suggested in human cells that Pol ε, is involved in DNA repair processes initiated by UV-damage. Human DNA polymerase ε and/or δ are required in the repair DNA synthesis step of nucleotide excision repair *in vitro*. It was also shown that the recombinational repair pathway, which repairs double strand breaks, requires both DNA polymerase δ and ε. *In vitro* studies have shown that crude extracts of temperature-sensitive *pol2* mutant cells have reduced activity in nucleotide excision repair and base excision repair. However, the suggestion that Pol ε functions in DNA repair has been questioned based on the observation that several mutant *pol2* alleles that have temperature-sensitive Pol ε activity *in vitro* do not show any significant sensitivity to DNA-damaging reagents. Since most of the evidence that Pol ε participates in DNA repair and recombination derives from *in vitro* experiments, further studies are required to confirm that this is also the case *in vivo*.

## Role in Checkpoint Control

In order to prevent premature mitosis before the whole genome is completely duplicated, the S phase checkpoint is activated when S phase progression is inhibited, for example, by DNA damage. This process involves a protein phosphorylation signal cascade, slowing down of DNA synthesis, and induced transcription of certain genes. Several mutations that cannot induce checkpoint-dependent transcription after DNA damage have been isolated and characterized in budding yeast. One of these mutations is located in the carboxyl-terminal region of the *POL2* gene, suggesting that Pol ε may act as a DNA-damage "sensor" that triggers expression of checkpoint and repair genes. Additional evidence for this view is provided by the observation that Pol ε binds to a factor which participates in S phase checkpoint control under certain conditions.

### See Also the Following Articles

Cell Cycle: DNA Damage Checkpoints • Cell Cycle: Mitotic Checkpoint • DNA Mismatch Repair in Mammals • DNA Polymerase α, Eukaryotic • DNA Polymerase β, Eukaryotic • DNA Polymerase δ, Eukaryotic • DNA Polymerases: Kinetics and Mechanism • DNA Replication Fork, Eukaryotic • Translesion DNA Polymerases, Eukaryotic • Zinc Fingers

### Glossary

**B family DNA polymerase** DNA polymerases in bacteria, archaea, and eukarya are classified into six groups (A, B, C, D, X, and Y) based on their amino acid sequences.

**exonuclease activity** An activity which digests single-stranded DNA from an end. The direction of exonuclease activity is specific: 5′–3′ or 3′–5′.

**replication fork** During DNA replication, the template strands of the duplex DNA separate to form a replication fork. Leading strand is continuously synthesized and lagging strand is synthesized discontinuously.

**zinc-finger motif** Amino acid sequences that bind zinc, composed with two repeats of two closely spaced cysteine or histidine. It mediates protein–protein interaction or protein–DNA interaction.

## Further Reading

Kawasaki, Y., and Sugino, A. (2001). Yeast replicative DNA polymerases and their role at the replication fork. *Molecul. Cells* **12**, 277–285.

Newlon, C. S. (1996). DNA replication in yeast. In *DNA Replication in Eukaryotic Cells* (M. L. DePamphilis, ed.) pp. 873–914. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Wang, T. S.-F. (1996). Cellular DNA polymerases. In *DNA Replication in Eukaryotic Cells* (M. L. DePamphilis, ed.) pp. 461–493. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

## Biography

Yasuo Kawasaki is an Assistant Professor of the Graduate School of Frontier Biosciences at Osaka University in Osaka, Japan. His principal research interests are in the regulation of S phase and the mechanism of chromosomal DNA replication in eukaryotes. He holds a Ph.D. from Kyoto University and received his postdoctoral training at Cornell University. He is actively studying the regulation and mechanism of chromosomal DNA replication in budding yeast *Saccharomyces cerevisiae*.

Akio Sugino is a Professor of the Graduate School of Frontier Biosciences at Osaka University in Osaka, Japan. His principal research interests are in the regulation of S phase and characterization of DNA polymerases in eukaryotes. He holds a Ph.D. from Nagoya University. He started his research career with Professor Reiji Okazaki and participated in the works for finding the discontinuous DNA replication of the lagging strand synthesis.

# DNA Polymerase I, Bacterial

**Catherine M. Joyce**
*Yale University, New Haven, Connecticut, USA*

**DNA polymerase I is the most abundant DNA polymerase in eubacteria. Although it lacks the speed and processivity of the more complex polymerases which replicate the bacterial chromosome, it is ideally suited for the synthesis of short stretches of DNA in excision repair and in the removal of RNA primers during lagging strand replication. DNA polymerase I (pol I) of *Escherichia coli*, the first DNA polymerase to be discovered and studied, has long served as the prototype for this class of enzymes. Other homologues that have been extensively studied are the thermostable DNA polymerase I from *Thermus aquaticus* (*Taq* DNA pol) and the DNA polymerase of bacteriophage T7.**

## Overview of Structure

Pol I enzymes are multifunctional and follow the pattern seen throughout the polymerase superfamily: a modular structure with a common polymerase domain (described below) and auxiliary enzyme activities located on separate protein domains. All bacterial pol Is have an N-terminal domain with 5′ nuclease activity; this activity is responsible for the removal of DNA ahead of the growing primer strand. Attachment of the 5′ nuclease domain to the rest of the polymerase is protease sensitive; in *E. coli* pol I mild protease digestion was originally used to remove the 5′ nuclease (35 kDa) from the remainder of the molecule (called Klenow fragment, 68 kDa); nowadays this is accomplished by recombinant DNA manipulations. The linkage between the 5′ nuclease and Klenow fragment is almost certainly flexible, as shown by the observation that the 5′ nuclease does not occupy the same position relative to the rest of the molecule in different crystal forms of *Taq* DNA pol. The pol I homologues found in bacteriophage genomes resemble the Klenow fragment portion of the bacterial pol Is, in that they lack the 5′ nuclease portion of the sequence. In some cases, such as bacteriophage T7, the 5′ nuclease is encoded as a separate gene product.

The Klenow fragment portion of pol I also contains two domains. In about half of the bacterial pol Is, the N terminal of the two domains contains a 3′–5′ exonuclease (3′ exo) activity which serves as a proofreader to eliminate polymerase errors. If the polymerase were to insert an incorrect nucleotide, resulting in a terminal mispair, the 3′–5′ nuclease would remove the incorrect primer-terminal nucleotide and provide a second opportunity for correct insertion. The bacterial pol I enzymes that lack 3′–5′ exonuclease activity (from species including *Thermus*, *Bacillus*, and *Rickettsiae*) nevertheless contain the domain, though key active site side chains are absent. Aside from the thermophiles, where fraying of double-stranded DNA termini at high temperatures might encourage excessive nuclease degradation, it is unclear why certain bacterial pol I enzymes have proofreading activity and others do not.

## Polymerase

### Domain Structure and Relation to Other Polymerases

When the structure of Klenow fragment (the first polymerase to be studied crystallographically) was reported in 1985, the polymerase domain structure was described as resembling a half-open right hand, with subdomains called “fingers,” “palm,” and “thumb” forming a cleft. Currently, structures are available for more than 30 polymerases, encompassing all four biochemical categories of polymerase (DNA or RNA dependent, with DNA or RNA as product), and it is clear that the domain structure originally observed in Klenow fragment is a common theme (Figure 1). The palm subdomain is the most highly conserved across polymerase families; it contains a conserved structural motif (the “polymerase fold”) consisting of a three-stranded antiparallel $\beta$-sheet supported by two $\alpha$-helices. This serves as the scaffold for important active site residues, in particular a pair of carboxylate ligands to the two divalent metal ions that catalyze the phosphoryl transfer reaction. The fingers and thumb subdomains are structurally much more divergent, but carry out analogous functions in all polymerases – the fingers providing important active site residues, particularly those involved in nucleotide binding, and the thumb binding the primer-template duplex.

 © 2004, Elsevier Inc. All Rights Reserved. 

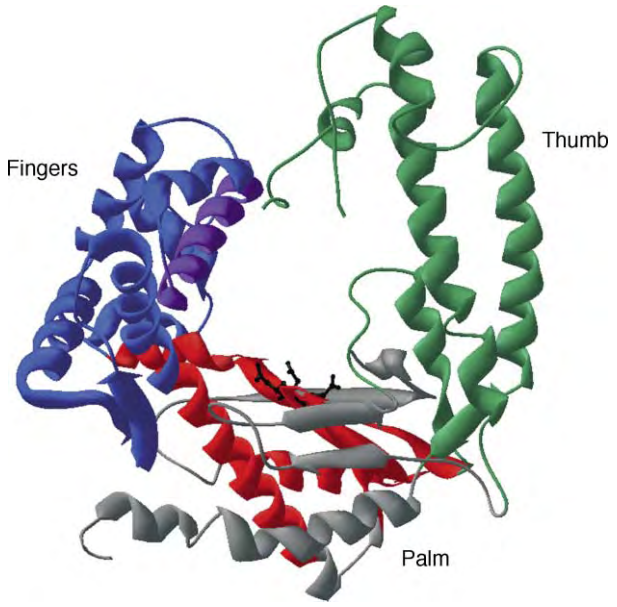



**FIGURE 1** Three-dimensional structure of the polymerase domain of *E. coli* DNA polymerase I (from the work of T. A. Steitz and colleagues). The thumb and fingers subdomains are colored green and blue, respectively, with the O-helix on the fingers subdomain in purple. The palm subdomain is gray, except for the "polymerase fold," a conserved structural motif found in the majority of nucleic acid polymerases, which is shown in red. Three conserved carboxylate side chains, two of which are ligands to the catalytic metal ions, mark the location of the polymerase active site.

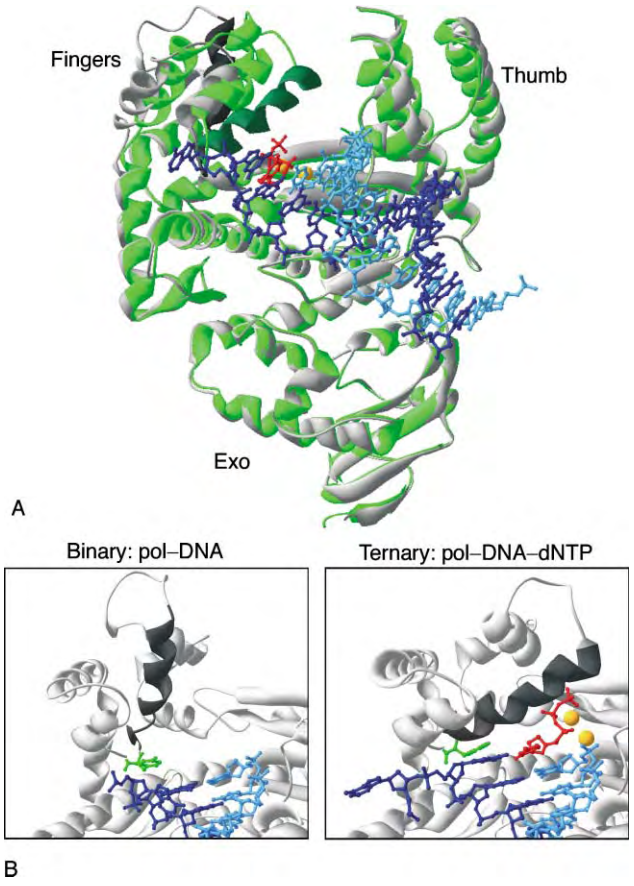



**FIGURE 2** (A) Superposition of binary (pol–DNA) and ternary (pol–DNA–dNTP) complexes of Klentaq, the Klenow fragment portion of *Taq* DNA pol (from the work of G. Waksman and colleagues). The DNA template (dark blue) and primer (cyan), incoming nucleotide (red) and active-site metal ions (gold) are shown in their positions in the ternary complex. The protein backbone in the binary complex is gray, and in the ternary complex green. The two protein structures are essentially superimposable except within the fingers subdomain, where the largest movement involves the O-helix (shown in darker shades of gray and green, respectively). The domain marked "Exo" corresponds to the 3′–5′ exonuclease domain in Klenow fragment, though it has no enzymatic activity in Klentaq. (B) Detailed comparison of the active site regions in the two complexes. In each case the protein is shown in gray, with the O-helix in a darker shade. Coloring of the DNA, nucleotide, and metal ions is the same as in the top panel. The structures are oriented so that the palm subdomain and the DNA are in similar positions in both panels, emphasizing the movement of the O-helix. The side chain of the conserved Tyr at the C-terminus of the O-helix (Tyr671 in Klentaq, Tyr 766 in Klenow fragment) is shown in green. This side chain is stacked with the template side of the terminal base pair in the binary complex; in the ternary complex it has moved out of the way to allow the next template base to enter the active site (note that this template base is folded back over the template strand in the binary complex). For clarity, the top of the thumb subdomain is omitted in all the structures illustrated in this figure.

## Substrate Binding

The binding of substrates at the polymerase active site has been revealed in cocrystals of several pol I homologues. The template-primer duplex (corresponding to the product of DNA synthesis) is bound in a shallow cleft between the thumb and 3′ exo domains, with the largely $\alpha$-helical thumb providing important binding contacts to the phosphate backbone. In a polymerase–DNA binary complex, the primer terminal base pair abuts the side of the polymerase cleft formed by the fingers subdomain. This wall, which defines one side of the active site, is formed primarily by a long $\alpha$-helix (the O-helix, shown in purple in Figure 1) which runs the length of the fingers subdomain and has a group of important and highly conserved side chains on the surface pointing into the cleft. At the C terminus of the O-helix is an invariant Tyr side chain which is stacked against the template side of the terminal base pair.

When the dNTP complementary to the templating position is added so as to form a polymerase–DNA–dNTP ternary complex, cocrystal structures indicate that the polymerase must have undergone a conformational change (Figure 2). The novel conformation seen in ternary complex structures differs from the apoenzyme and binary complex structures by a substantial movement of the fingers subdomain so as to close the polymerase cleft. In conjunction with the transition from the open (binary complex) to closed (ternary complex) conformation, the Tyr side chain at the C-terminus of the O-helix moves further down into

the active site, away from its stacked position with the terminal base pair. This allows the templating base to stack with its 3′ neighbor at the duplex terminus, and to base pair with the incoming dNTP in a snug binding pocket formed by side chains of the O-helix and nearby residues on one side and the primer-terminal base pair on the other side. Analogous conformational transitions have been inferred from crystal structures of a diverse variety of polymerases, suggesting that the formation of a closed ternary complex may be a common feature of the polymerase reaction pathway.

## DNA Polymerase Reaction Mechanism

Extensive work by Benkovic and co-workers has defined the polymerase reaction pathway of Klenow fragment. There is an obligatory order of substrate binding – DNA before dNTP – as would be expected, given that templating information is required for nucleotide selection. As mentioned above, the chemical step of dNTP addition is catalyzed by two divalent metal ions coordinated at the active site (Figure 3). One metal ion facilitates deprotonation of the primer 3′-OH, making it a better nucleophile, while the other stabilizes the pyrophosphate leaving group. Both metals also stabilize the developing negative charge on the $\alpha$-phosphate in the transition state. This two-metal-catalyzed reaction mechanism is beautifully illustrated in the ternary complex cocrystal structures, and is widespread in phosphoryl transfer reactions, including the 3′–5′ exonuclease reaction (discussed below). The work of Benkovic and co-workers indicates that the phosphoryl transfer step of Klenow fragment is rapid, but is preceded and followed by slow noncovalent steps. These steps must correspond to some kind of conformational transition and it is tempting to equate the step preceding phosphoryl transfer with formation of the closed complex inferred from the crystal structures. However, it is important to realize that there is currently no experimental evidence in support of this idea.

## DNA Polymerase Reaction Specificity

### Base Pairing

DNA polymerases function with extraordinary specificity, selecting the complementary dNTP and rejecting incorrect choices in the majority of cases. Klenow fragment makes approximately one error for every $10^5$ nucleotides synthesized, about 1000-fold more accurate than would be predicted solely from the energetics of base pairing. Three major mechanisms have been proposed to account for the way in which the polymerase enhances the accuracy of DNA synthesis.

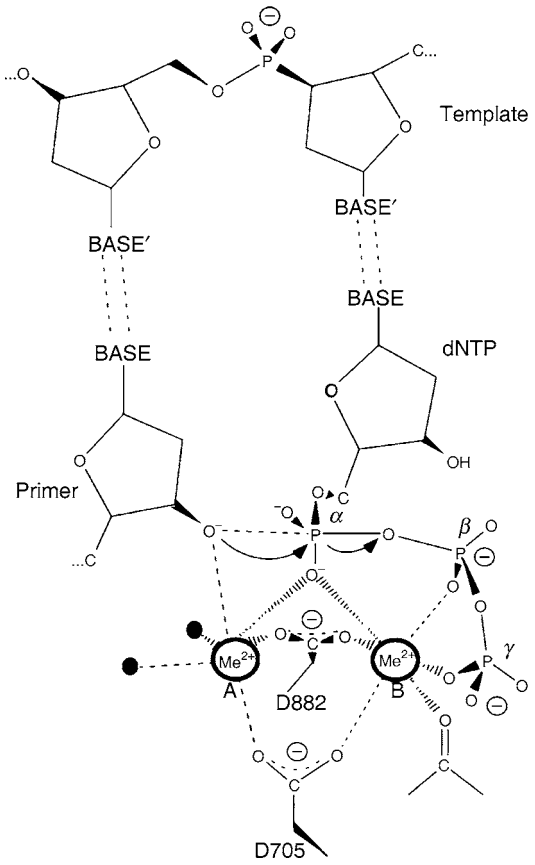



**FIGURE 3** Mechanism of phosphoryl transfer, resulting in nucleotide addition, at the polymerase active site. Catalysis is mediated by two divalent metal ions, coordinated, in Klenow fragment, by Asp705 and Asp882. Metal ion A activates the primer 3′-OH for nucleophilic attack on the $\alpha$-phosphate of the dNTP. Metal ion B stabilizes the negative charge that develops on the pyrophosphate leaving group. Both metal ions also assist the reaction by stabilizing the negative charge on the $\alpha$-phosphate in the pentacovalent transition state. Adapted from Brautigam and Steitz (1998).

The first is exclusion of water from the active site which should amplify the energetic differences between correct and incorrect base pairings. The second is geometric selection in the binding pocket for the nascent base pair such that there is good steric complementarity to the rather symmetrical shape of a Watson–Crick base pair, but exclusion of mispairs. This model is consistent with the very snug fit seen in the binding pocket of polymerase ternary complex structures. Moreover, several polymerases, including Klenow fragment, have been shown to incorporate template-primer pairings that mimic the shape of a Watson–Crick base pair but lack any hydrogen bonds. These first two specificity mechanisms are proposed to operate at the level of nucleotide addition, and should weaken the binding or slow the incorporation of an incorrectly paired incoming nucleotide. The third selection method would operate after nucleotide addition and relies on hydrogen-bonding interactions between the polymerase and

the minor groove at or close to the primer terminus. The hydrogen-bond acceptors on N3 of purines and O2 of pyrimidines occupy similar positions in all Watson–Crick base pairs but are mispositioned in incorrect pairings, thus providing a mechanism for scanning the minor groove to ensure that template information has been correctly read. A mispair located at the primer terminus will fail to make the minor groove interactions that are seen for correct base pairs in polymerase cocrystal structures, and this, together with other geometric abnormalities, results in a much slower rate of addition of the next nucleotide. Slower polymerase addition to a mispaired primer terminus reduces the likelihood of a mispair becoming fixed as a mutation by continued synthesis, and also increases the time window during which the error may be proofread, or the mispaired DNA may dissociate.

As might be expected from the different geometries of various mispairs, a polymerase such as Klenow fragment does not make all errors at the same frequency, so that insertion of dGTP opposite a template T is relatively facile, whereas insertion of dCTP opposite C is rare. Klenow fragment mutator mutants change not only the frequency of errors but also the specificity, and may provide a valuable window into the recognition processes that take place at the polymerase active site.

### Sugar Specificity

Bacterial pol Is also have stringent specificity for the sugar of the incoming nucleotide. A DNA polymerase must reject ribonucleotides, despite their higher concentration *in vivo*, and this is achieved by an invariant Glu residue positioned so as to obstruct any substituent on the C2′ position of the sugar. Mutation of this Glu to the smaller Ala in Klenow fragment results in a polymerase that can add a ribonucleotide almost as easily as a deoxyribonucleotide. However, the mutant enzyme does not function as a true RNA polymerase because it cannot efficiently add multiple ribonucleotides, most probably because the duplex binding site cannot accommodate a DNA–RNA product.

Pol I enzymes fall into two classes regarding their handling of nucleotide analogues which lack the sugar 3′ hydroxyl (dideoxy nucleotides) and therefore function as chain terminators. The majority, exemplified by Klenow fragment, discriminate very strongly against dideoxy nucleotides. The ternary complex cocrystal structures show a hydrogen bond between the 3′ hydroxyl and the $\beta$-phosphate of the incoming nucleotide; presumably, the loss of this interaction compromises the alignment of reactive groups in the transition state, resulting in a slower reaction rate. A minority of pol Is, exemplified by T7 DNA pol, discriminate only slightly against dideoxy nucleotides. In these polymerases, a conserved aromatic side chain that forms part of the nucleotide binding pocket is present as Tyr, instead of the more usual Phe, and the Tyr hydroxyl provides the missing interaction with the $\beta$-phosphate. The ability to manipulate chain terminator specificity via a single active-site point mutation has proven invaluable in DNA sequencing and related biotechnology applications.

## 3′–5′ Exonuclease

The 3′–5′ exonuclease, present in about half of pol Is, carries out its editing function by means of hydrolysis of the terminal nucleotide of the DNA primer strand. The hydrolysis reaction is a phosphoryl transfer, analogous to that described above for the polymerase reaction, catalyzed by a pair of metal ions liganded by a cluster of conserved carboxylate side chains. The other important component of the 3′ exo site is a binding site for single-stranded DNA. In model studies, the exonuclease can be studied using single-stranded DNA as the substrate, though the natural substrate *in vivo* is a duplex DNA whose primer terminus is frayed so as to present three or four bases of single-stranded DNA for binding at the 3′ exo site. The requirement for fraying in order to bind at the 3′ exo active site provides the specificity for editing, because a mismatched primer terminus is more easily melted and is therefore a better substrate for the 3′ exo than a correctly paired DNA. Indeed, studies have shown that, whereas a correctly paired DNA duplex is bound predominantly at the polymerase site of Klenow fragment, a single terminal mismatch results in $\approx$50:50 partitioning between polymerase and 3′ exo sites. The preference of the 3′ exo for a mispaired substrate is amplified by the slowing of the polymerase reaction caused by a mispaired primer terminus (discussed above), so that a mispair is likely to be targeted for proofreading, while a correct primer terminus will serve as a substrate for continued addition.

The contribution of proofreading to polymerase fidelity is quite variable in the pol Is. As already noted, some pol Is do not proofread at all. In others, such as *E. coli* pol I, the contribution of proofreading is relatively modest, about tenfold. Others, such as the polymerases from bacteriophages T5 and T7, have more active exonucleases. Additionally, depending on the strength of the polymerase–DNA interaction, the DNA may stay associated with the polymerase during the transfer from polymerase to editing site, as in T5 and T7, or it may reach the proofreading site via dissociation from the polymerase site and binding to the exonuclease site of another enzyme molecule. Because the selectivity of editing is determined by the structure of the DNA substrate (paired or mispaired) and does not require presentation of the primer terminus in a particular way, the intermolecular transit from polymerase to

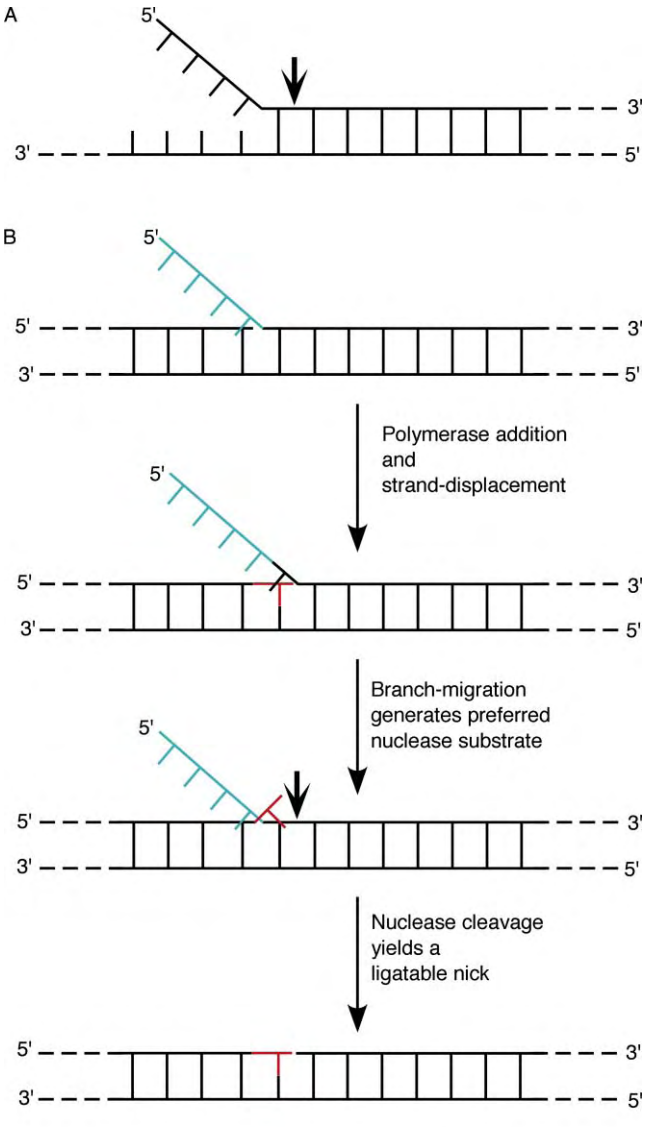



**FIGURE 4** (A) Site of cleavage by the 5′ nuclease on a model substrate containing noncomplementary 5′ and 3′ extensions. The nuclease cuts between the first two paired bases. (B) The polymerase and 5′ nuclease activities collaborate in processing a DNA molecule with a 5′ single-stranded tail so as to produce a nicked product that can be sealed by DNA ligase. Cleavage of the first structure, between the first two paired bases adjacent to the 5′ tail, would yield a single-base gap. Nucleotide addition by the polymerase (in red), together with the preference of the 5′ nuclease for a melted base at the 3′ end of the adjacent primer strand, favors the formation of a ligatable product.

exonuclease site would not be expected to compromise the editing process.

## 5′ Nuclease

The 5′ nuclease of the bacterial pol I enzymes was described as the "5′–3′ exonuclease" in the earlier literature. However, it is now recognized that this activity is not a true exonuclease, but is a structure-specific nuclease that recognizes the junction between duplex DNA and a single-stranded 5′ end, and cuts between the first two paired bases (Figure 4A). On a nicked DNA molecule, polymerase-catalyzed synthesis causes strand displacement and generates the substrate for the 5′ nuclease, so that together the polymerase and 5′ nuclease cause "nick translation," the movement of a nick along a duplex DNA molecule. DNA binding experiments show that the polymerase and 5′ nuclease compete for binding to a DNA substrate, and this implies a mechanism in which the DNA is passed from one active site to the other, and rules out mechanisms in which both reactions take place within a single bound species. The preferred substrate for the 5′ nuclease has, in addition to the unpaired 5′ strand, an unpaired base at the 3′ end of the primer strand abutting the ss–ds junction. This substrate could be formed by rearrangement of the product of polymerase extension and, when cleaved by the 5′ nuclease, will leave a junction ready for ligation (see Figure 4B). The observed specificity of the 5′ nuclease fits well with the *in vivo* functions of the bacterial pol I enzymes in DNA repair and in the removal of primers from Okazaki fragments on the lagging strand. In both cases, the desired endpoint of pol I action is a nick that can be sealed by DNA ligase.

Several 5′ nuclease structures have been solved in the absence of substrates, but our structural understanding of the 5′ nuclease is less good than for the polymerase or 3′–5′ exonuclease because of the absence of cocrystal structures. The location of the 5′ nuclease active site has been inferred from the position of conserved sequence motifs. These contain a large number of conserved carboxylate residues, inviting speculation that, like the polymerase and 3′–5′ exonuclease activities, the 5′ nuclease may carry out phosphoryl transfer using bound divalent metal ions. The bacterial 5′ nucleases and their archaeal homologues have a protein loop or arch in their structures, and it has been hypothesized that the single-stranded 5′ end of the DNA substrate is threaded through the protein.

## See Also the Following Articles

DNA Polymerase II, Bacterial • DNA Polymerase III, Bacterial • DNA Polymerases: Kinetics and Mechanism • Exonucleases, Bacterial • umuC,D Lesion Bypass DNA Polymerase V

## Glossary

**apo-enzyme** Enzyme in the absence of bound substrates.
**mispair** Two opposed bases in DNA that do not conform to the Watson–Crick rules of complementarity, i.e., not A–T or G–C.
**primer** In duplex DNA, the strand that presents a 3′ end for extension by a DNA polymerase.

**template** In duplex DNA, the strand that is paired with the primer, and therefore provides the information for copying by DNA polymerase.

## Further Reading

Benkovic, S. J., and Cameron, C. E. (1995). Kinetic analysis of nucleotide incorporation and misincorporation by Klenow fragment of *Escherichia coli* DNA polymerase I. *Methods Enzymol.* **262**, 257–269.

Brautigam, C. A., and Steitz, T. A. (1998). Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. *Curr. Opin. Struct. Biol.* **8**, 54–63.

Ceska, T. A., and Sayers, J. R. (1998). Structure-specific DNA cleavage by 5′ nucleases. *Trends Biochem. Sci.* **23**, 331–336.

Joyce, C. M. (1997). Choosing the right sugar: How polymerases select a nucleotide substrate. *Proc. Natl Acad. Sci. USA* **94**, 1619–1622.

Kunkel, T. A., and Bebenek, K. (2000). DNA replication fidelity. *Annu. Rev. Biochem.* **69**, 497–529.

Li, Y., Korolev, S., and Waksman, G. (1998). Crystal structures of open and closed forms of binary and ternary complexes of the large fragment of *Thermus aquaticus* DNA polymerase I. Structural basis for nucleotide incorporation. *EMBO J.* **17**, 7514–7525.

## Biography

Catherine M. Joyce is a Senior Research Scientist in the Department of Molecular Biophysics and Biochemistry at Yale University. Her research is directed toward a structural interpretation of DNA polymerase reaction mechanisms.

# DNA Polymerase II, Bacterial

**Judith L. Campbell**
*California Institute of Technology, Pasadena, California, USA*

**DNA must be synthesized during cellular reproduction to produce an identical copy of the chromosome, a process known as DNA replication. In addition, DNA synthesis is needed during the process of DNA repair. After DNA damage is removed from one strand, the complementary strand can serve as a template for correct resynthesis of an intact chain. DNA is synthesized from deoxynucleoside triphosphate building blocks through the action of enzymes called DNA polymerases. All cells contain several DNA polymerases, each of which is specialized either for DNA replication or for different repair pathways. *Escherichia coli* contains five DNA polymerases. DNA polymerase III is essential for DNA replication, while the remaining DNA polymerases are for DNA repair. The specific DNA polymerase being recruited to sites of DNA damage depends on a number of factors. DNA polymerase II, so-called because it was the second DNA polymerase described in *E. coli*, is specialized for DNA repair that occurs when moving replication forks encounter damaged DNA templates.**

## DNA Polymerase II: Structure and Biochemical Functions

DNA polymerase II (pol II) is a single polypeptide of 783 amino acids with a predicted mass of 89.9 kDa, and is encoded by the *polB* (a.k.a. *dinA*) gene. DNA pol II is the founding member of the B family of DNA polymerase structures, and contains the five motifs characteristic of this family, including both DNA polymerase and exonuclease domains. (DNA pol I belongs to family A, DNA pol III to family C, and DNA poly IV and V to family Y.) Members of the family B polymerases are found throughout the Archaea; and, interestingly, the three replicative DNA polymerases of eukaryotic cells, as well as additional repair DNA polymerases, belong to family B. This suggests the evolutionary importance of pol II.

DNA pol II is a monomeric enzyme but interacts with accessory subunits of the pol III replicase, the $\gamma$–$\delta$ clamp loading complex and the $\beta$ clamp. The clamp tethers the DNA polymerase to the terminus of the growing DNA chain. These subunits confer high processivity on both enzymes, a property consistent with a role in synthesizing long stretches of DNA, either during repair or replication. In fact, all five bacterial polymerases interact with the $\beta$-clamp. This interaction is competitive, suggesting that all polymerases use the same interface on the $\beta$-clamp. This observation suggests a mechanism by which the polymerases may be transiently interchanged with the replicase to copy sites in the template that pose blocks to pol III, such as modification of the bases, bound proteins, or sequences that are inherently inhibitory to polymerase translocation because of their structure. This polymerase switching phenomenon is important for the coordination of DNA replication and DNA repair, and thus for the maintenance of DNA synthesis fidelity.

In addition to being capable of highly processive DNA synthesis, DNA pol II is highly accurate, with *in vitro* error rate as low as one in a million. Mutations in the $3'$–$5'$ proofreading exonuclease domain of the pol II protein result in a 13–240-fold increase in the error rate, depending on the type of error being edited. *In vivo*, defects resulting in a 1000-fold reduction in the proofreading exonuclease activity of pol II cause no increase in mutation frequency in cells containing normal DNA pol III. When an antimutator allele of DNA pol III was present in the cells, however, the pol II proofreading mutant experienced a significant increase in chromosomal base substitution and frameshift mutation frequencies in dividing cells. This suggests that, while pol III is the normal replicase, pol II can replace pol III at the replication fork under some circumstances. In non-dividing cells, pol II exonuclease-defective mutants and mutants completely lacking pol II due to deletion of the *polB* gene have a mutator activity for adaptive mutation. (Adaptive mutations in *E. coli* are episomal mutations arising in stationary phase, nongrowing cells, and the highly conserved pol IV is thought to introduce the mutations during synthesis initiated at the conjugal origin or by DNA repair and recombination.) Thus, an additional role of the pol II exonuclease activity may be to control the level of adaptive mutation by pol IV.

 © 2004, Elsevier Inc. All Rights Reserved. 

## Biological Function: Replication Restart

Given that pol II is a high-fidelity polymerase and that it is highly processive in the presence of the $\beta$-clamp, pol II has properties characteristic of replicative DNA polymerases, i.e., ability to synthesize long stretches of DNA at high rates and with high fidelity. However, deletion of pol II from the cell is not deleterious to growth of *E. coli* cells, suggesting that the polymerase is present in order to carry out repair of damaged DNA. This appears to be the case, since the levels of pol II are induced sevenfold by treatment of cells with UV light, which causes lesions in the DNA. In fact, pol II is one of ~40 genes that comprises of the SOS regulon. SOS is a global response to DNA damage that induces ~40 genes, ~30 of which, like *polB*, are induced specifically by inactivating a repressor known as LexA. The level of pol II rises from ~50 molecules in normally replicating cells to ~350 after derepression. Two additional DNA polymerases (pol V and pol IV), both of which have low fidelity, are also induced after cells are treated with DNA-damaging agents.

Although the level of pol II increases in cells after DNA damage, *polB* mutants are not sensitive to DNA-damaging agents such as UV. This is because pol V can carry out repair in the absence of pol II. This conclusion derives from the observations that pol V mutants are UV sensitive and mutants lacking both pol V and pol II are even more so. The mechanism of repair catalyzed by the two polymerases is quite different, however. After treatment with UV, DNA replication is transiently inhibited. Within ~10–15 min, DNA replication resumes, eventually returning to the normal rate. Pol II is induced 30 s after treatment with UV and is required for this replication restart, also known as replisome reactivation. During replisome reactivation, efficient repair mechanisms remove most of the DNA damage. Repair carried out in the presence of pol II is error free and does not give rise to increased mutation. The steps in error-free replication restart involving pol II are shown in Figure 1. When the replisome encounters damage in the template that blocks the progress of pol III, there is a rearrangement of the replication fork. The replication of the two complementary strands, which is usually concurrent, is uncoupled. Synthesis on the damaged template strand is blocked but synthesis on the undamaged strand can continue, resulting in faithful copying of the undamaged strand beyond the site of the lesion. Next, the nascent DNA chains unwind from the parental templates in a process of rotary diffusion called branch migration, and then reassociate with each other by virtue of their complementarity. The longer nascent strand then serves as a template for the shorter, previously blocked strand, which can now be extended past the site of the lesion using the undamaged, and therefore instructional, bases from the newly synthesized complementary strand. This synthesis is thought to occur by substituting pol II for pol III, perhaps via the $\beta$-clamp, as already suggested. This part of the reaction is sometimes called template switching. In order to recreate the rapidly moving replication fork, the process is then reversed. The nascent strands unwind and switch back to the parental template, restoring the replication fork at a position downstream of the lesion. As a result of these isomerizations, the lesion is effectively bypassed by the normal replication fork and moved into the fully duplex region upstream of the fork, where it can be recognized and dealt with by the various repair systems in the cell. To complete the process of replication restart, both kinetic and genetic evidence support the notion that after restoration of the replication fork, there is a switch back to

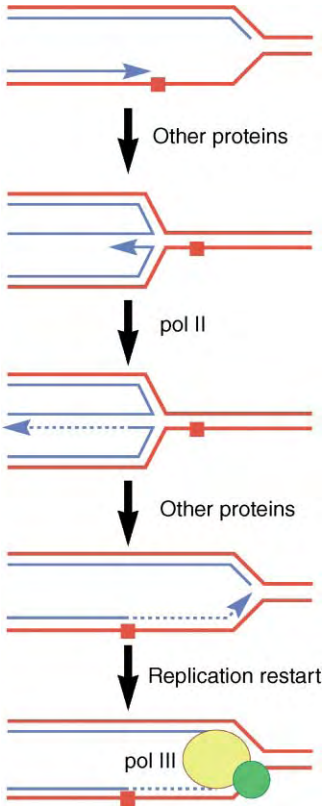



**FIGURE 1** A hypothetical scheme for replication restart using bacterial DNA polymerase II. The red lines represent template DNA, and the blue lines newly synthesized DNA. The arrow represents the end of the chain where the polymerase switch occurs. The dotted line represents a lesion in the DNA that cannot be copied by pol III. Pol III replicase, depicted as the yellow and green ovals, is shown after reassociation with the fork at the bottom of the figure. The various steps are described in the text in detail. (Modified from Rangarajan, S., Woodgate, R., and Goodman, M. F., (2002). Replication restart in UV-irradiated *Escherichia coli* involving pols II, III, V, PriA, RecA, and RecFOR proteins. *Mol. Microbiol.* **43**, 617–628, with permission of Blackwell Publishing Ltd.)

the pol III replicase in order to complete normal replication. Thus, pol II carries out only transient synthesis. Several proteins in addition to pol II, as indicated in Figure 1, genetically implicated in replication restart after UV irradiation, are likely to aid in the uncoupling, branch migration, and recruitment of pol II described in the model. Since the reaction has not been reconstituted from purified proteins, however, the molecular mechanism remains to some extent conjectural. One enigma with respect to the role of pol II in replication restart is the finding that levels of pol II are substantial even in cells lacking DNA damage. This has led to speculation that pol II might be involved in error-free replication restart in the presence of endogenous blocks to replication, such as oxidative damage due to normal metabolism, deamination of cytosine, or protein blocks.

In the absence of pol II, DNA replication is still inhibited immediately following DNA damage, but replication restart is delayed for ~50 min after UV irradiation. Biochemical studies suggest that pol V directly substitutes for pol III at the growing fork, and that pol V then inserts a base directly across from the lesion. This process is called translesion synthesis (TLS). After insertion, there is a switch back to pol III for continued replication. TLS is not only fundamentally different in mechanism from pol II catalyzed replisome reactivation, it also has a very different outcome for the surviving cells. Pol V has more relaxed base recognition properties than pol II or pol III, and can efficiently incorporate bases opposite certain lesions and abasic sites, thus giving rise to mutations primarily targeted to the lesions. Thus, repair in the absence of pol II is mutagenic and is therefore known as error-prone repair. Even in the presence of pol II, mutation frequency is induced 100-fold by UV irradiation of *E. coli* cells. It is reasonable that the pol II pathway temporally precedes TLS, since pol II-dependent replication restart does not increase the mutational load. Apparently, however, TLS is a fail-safe pathway which is employed because the organism prefers high survival rates, even with increased mutation load, pending return to an environment supporting more robust growth.

While the polymerase chosen for repair of damage that blocks replication forks depends on availability of polymerase (pol II must be induced), the repair pathway chosen also depends on the type of damage. In the case of N-2 acetylaminofluorene (AAF) guanine adducts, pol II acts as an error-prone TLS polymerase and generates frameshift mutations, while pol V carries out error-free translesion synthesis.

In summary, pol II can, under some conditions, replace pol III in chromosomal replication, but its primary role is in error-free replication restart in response to DNA damage.

## See Also the Following Articles

DNA Polymerase I, Bacterial • DNA Polymerase III, Bacterial • DNA Replication Fork, Bacterial

## Glossary

**adaptive mutation** Mutations that arise presumably during DNA repair in cells that are not otherwise undergoing DNA replication. In bacteria, such mutations have been shown to occur on episomes. This process is probably important in evolution.

**episome** An extrachromosomal DNA molecule, residing in a cell, that cannot replicate on its own but needs the proteins encoded by the cellular chromosome.

**processivity** With respect to DNA polymerases, the ability to copy long stretches of template without dissociating from the template.

**repressor** A protein that shuts down production of RNA from specific genes (i.e., turns them off). Repressors can be removed or inactivated by inducers.

## Further Reading

Campbell, J. L., Soll, L., and Richardson, C. C. (1972). Isolation and partial characterization of a mutant of *Escherichia coli* deficient in DNA polymerase II. *Proc. Natl Acad. Sci. USA* **69**, 2090–2094.

Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J., and Marians, K. J. (2000). The importance of repairing stalled replication forks. *Nature* **404**, 37–41.

Goodman, M. F. (2002). Error-prone repair DNA polymerases in prokaryotes and eukaryotes. *Annu. Rev. Biochem.* **71**, 17–50.

Hirota, Y., Gefter, M., and Mindich, L. (1972). A mutant of *Escherichia coli* defective in DNA polymerase II activity. *Proc. Natl Acad. Sci. USA* **69**, 3238–3242.

Knippers, R. (1970). DNA polymerase II. *Nature* **228**, 1050–1053.

Moses, R. E., and Richardson, C. C. (1970). A new DNA polymerase activity of *Escherichia coli*. II: Properties of the enzyme purified from wild-type *E. coli* and DNA-ts mutants. *Biochem. Biophys. Res. Commun.* **41**, 1565–1571.

Pham, P., Rangarajan, S., Woodgate, R., and Goodman, M. F. (2001). Roles of DNA polymerases V and II in SOS-induced error-prone and error-free repair in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **98**, 8350–8354.

Rangarajan, S., Woodgate, R., and Goodman, M. F. (1999). A phenotype for enigmatic DNA polymerase II: A pivotal role for pol II in replication restart in UV-irradiated *Escherichia coli*. *Proc. Natl Acad. Sci.USA* **96**, 9224–9229.

Rangarajan, S., Woodgate, R., and Goodman, M. F. (2002). Replication restart in UV-irradiated *Escherichia coli* involving pols II, III, V, PriA, RecA, and RecFOR proteins. *Mol. Microbiol.* **43**, 617–628.

## Biography

Dr. Judith Campbell is a Professor of Biology and Chemistry at the California Institute of Technology. In the 1970s her laboratory made contributions to the studies of the regulation of DNA replication of episomes in bacteria. Currently her laboratory studies include DNA replication and control of the cell cycle in the yeast *Saccharomyces cerevisiae*, with emphasis on DNA polymerases and DNA helicases.

# DNA Polymerase III, Bacterial

**Hisaji Maki**
*Nara Institute of Science and Technology, Ikoma, Japan*

**DNA polymerase III holoenzyme (pol III HE) is an enzyme that catalyzes elongation of DNA chains during bacterial chromosomal DNA replication. Bacterial cells contain several distinct DNA polymerases. In *Escherichia coli*, five DNA polymerases have been found and designated as DNA polymerase I to V in the order of their discovery. The main function of the third polymerase, pol III, is duplication of the chromosomal DNA, while other DNA polymerases are involved mostly in DNA repair and translesion DNA synthesis. Together with a DNA helicase and a primase, pol III HE participates in the replicative apparatus that acts at the replication fork. Unlike other bacterial DNA polymerases, pol III HE is a multisubunit complex, in which twin catalytic subassemblies, called the pol III core, are embedded with several other auxiliary subunits. Cooperative and coordinated action of these subunits enables pol III HE to function as the chromosomal replicase, concurrently synthesizing the leading and lagging strands of DNA. DNA synthesis by pol III HE is also characterized by a rapid chain-elongation reaction, high processivity, and high fidelity, all of which are essential for chromosomal DNA replication.**

## Replicative Apparatus in Bacterial Cells

In bacterial cells, the circular chromosome contains a unique origin, and DNA replication proceeds bidirectionally from the origin to the terminus. Replication of the whole bacterial genome (4700 kb for *E. coli*) is continuous from the origin to the terminus, and is accompanied by movement of the replicating point, called the replication fork. Both parental DNA strands are concurrently replicated at the fork. Since DNA polymerase can extend a DNA chain only in the $5' \rightarrow 3'$ direction, replication at a fork is semi-discontinuous: DNA synthesis is continuous on one strand (the leading strand) and discontinuous on the other (the lagging strand). Short pieces of DNA, called Okazaki fragments, are repeatedly synthesized on the lagging strand template. These Okazaki fragments are a few thousand nucleotides long in bacterial cells.

DNA replication is a complex process, involving numerous enzymes at the replication fork. In *E. coli*, more than 20 different proteins participate in DNA replication. Among these proteins, DnaB, DnaG, and pol III HE are the three basic components acting at the replication fork, forming a multiprotein complex called the "replisome" (Figure 1). The DnaB protein is a major replicative DNA helicase which moves along the lagging strand in the $5' \rightarrow 3'$ direction and opens up the duplex DNA at the tip of the replication fork to expose a pair of single-stranded DNA templates. The DnaG protein is a primase which synthesizes a short RNA to prime DNA chain elongation catalyzed by pol III HE.

The highly organized and remarkably efficient process needed for replicating chromosomal DNA is achieved by physical and functional interactions among these three components. Pol III HE associates with DnaB protein and pushes the helicase forward as it proceeds along the leading strand. Thus, the velocity of replication fork movement is determined mainly by the rate of chain elongation by pol III HE, $\sim$1000 bp $s^{-1}$. DnaB interacts with DnaG and activates cyclically the primase activity as the helicase moves along the lagging strand, resulting in timely initiation of Okazaki fragment synthesis. Finally, the proper recruitment of pol III HE to the replisome requires an RNA primer formed within the origin and, thus, depends upon the function of DnaG.

In bacterial cells, pol III HE and the other components of the replisome localize to discrete positions, predominantly at or near the midcell throughout the cell cycle. It is thought that the DNA is threaded through the centrally positioned replisome during duplication, and then extruded from the replisome after duplication.

## Subunits and Subassembly of Pol III Holoenzyme

The machine-like action of pol III HE at the replication fork, far more complex than what is necessary for mere DNA polymerization, is based on its subunit structure. Ten different polypeptides form an isolable 17-subunit pol III HE complex in *E. coli*. The whole assembly is very stable while participating in the replisome at

 © 2004, Elsevier Inc. All Rights Reserved. 

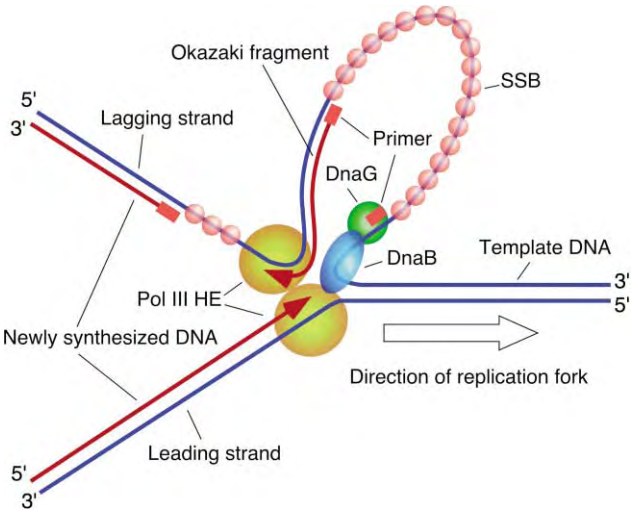



**FIGURE 1** The replication fork and three basic components of the replicative apparatus in bacteria.

the replication fork. However, during the course of purification, pol III HE readily disassembles into free subunits and various subassemblies, analyses of which have greatly helped to clarify the biochemical function of each subunit and the architecture of the holoenzyme (Figure 2). Smaller numbers of subunits assemble to form the holoenzyme in *Bacillus subtilis* and other bacterial species, although the basic architecture is conserved in most bacteria.

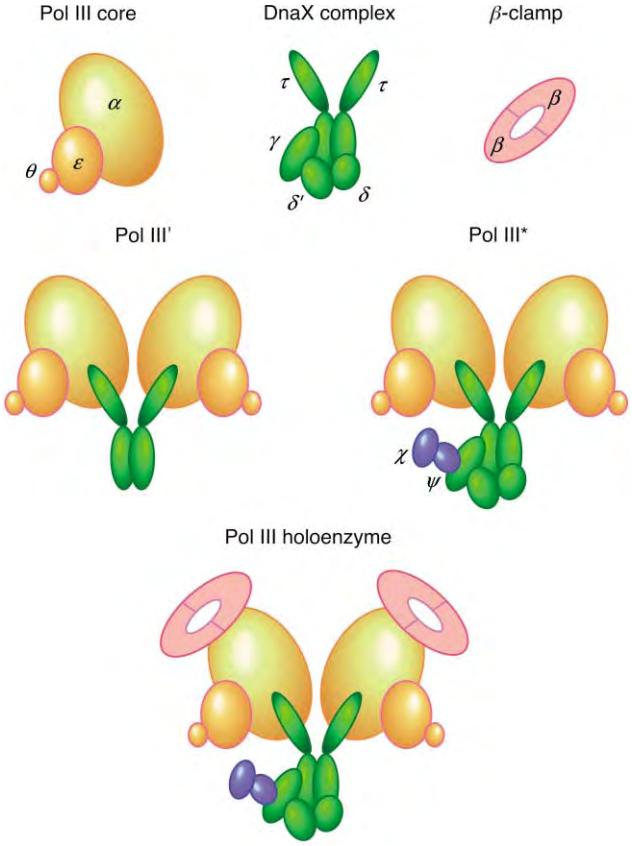



**FIGURE 2** A schematic view of the architecture of DNA polymerase III holoenzyme.

## Pol III Core

The $\alpha$-subunit, the largest polypeptide among the holoenzyme subunits, possesses DNA polymerase activity and forms a catalytic core subassembly, called the pol III core, with one molecule each of the $\varepsilon$ and $\theta$ subunits. The $\alpha$-subunit shares an amino-acid-sequence motif with bacterial DNA polymerases I and II, and with eukaryotic DNA polymerases $\alpha$, $\delta$, $\varepsilon$, and $\zeta$, which consists of six segments each containing 5–10 residues. This polymerase motif corresponds to catalytically important structural determinants for the active center of DNA polymerases. Based on small differences in the sequences of this motif, the DNA polymerases are divided into three classes: type A (pol I), type B (pol II and catalytic subunits of the eukaryotic polymerases), and type C ($\alpha$-subunit of pol III). In contrast to the type A and type B polymerases, the $\alpha$-subunit lacks the $3' \rightarrow 5'$ exonuclease activity required for the proofreading function, which removes nucleotides that have been incorrectly inserted by the polymerase. In the pol III core, the $\varepsilon$-subunit acts as the editing exonuclease and ensures highly accurate DNA synthesis by the polymerase. Cells that are defective in $\varepsilon$-subunit function show a strong mutator phenotype, with remarkably elevated frequencies of spontaneous mutation.

Although the pol III core has high catalytic efficiencies for DNA synthesis and proofreading, it cannot extend more than 10 nucleotide residues at once. This relatively low processivity of DNA synthesis by the pol III core is due to a low affinity of the core subassembly for template-primer DNA. Other subunits, especially the $\beta$-subunit, greatly enhance and regulate the processivity of the core.

## $\tau$-Subunit and Pol III′

The $\tau$-subunit is the second largest subunit, and forms a stable homodimer complex when it is free in solution. An important function of the $\tau$-subunit is to make the pol III core into a dimer. The C-terminal domain of the $\tau$-subunit interacts with the $\alpha$-subunit, and the dimeric nature of the $\tau$-subunit itself facilitates dimerization of the pol III core. The resulting subassembly, called pol III′, consists of two pol III cores and two $\tau$-subunits. The $\tau$-subunit improves the low processivity displayed by the pol III core, resulting in a moderately increased processivity for pol III′.

Besides dimerization of the pol III core, the $\tau$-subunit plays multiple roles at the replication fork. In the HE complex, the $\tau$-dimer bridges between dimeric polymerases and other auxiliary subunits. In addition, as

a scaffold of the replisome, the $\tau$-dimer connects pol III HE and the DnaB helicase at the replication fork.

### $\beta$-Subunit and Pol III*

Among the holoenzyme subunits, the $\beta$-subunit most easily dissociates from the holoenzyme complex. Phosphocellulose or cation-exchange column chromatography effectively separates the holoenzyme into the $\beta$-subunit and pol III*, a subassembly that retains all the subunits except $\beta$. However, the association of the $\beta$-subunit with pol III* is very stable when they form a ternary complex with template-primer DNA.

The $\beta$-subunit is a crescent-shaped polypeptide, and forms a homodimer with a doughnut-like structure which encircles double-stranded DNA and slides along the DNA. The $\beta$-dimer binds to the $\alpha$-subunit, even in the absence of other HE-subunits, and it tethers the $\alpha$-subunit to template DNA during DNA synthesis. The sliding-clamp nature of the $\beta$-subunit and the $\alpha$–$\beta$ interaction are the basis of the ability of pol III HE to perform highly processive DNA synthesis. The $\beta$-subunit also interacts with the $\delta$-subunit in an ATP-dependent manner when $\beta$ is functioning in the HE complex. Albeit much more weakly than its interaction with the $\alpha$-subunit, the $\beta$-dimer can associate with other proteins including all the other *E. coli* DNA polymerases, a mismatch repair protein (MutS), DNA ligase, and a replication initiator protein (DnaA). Involvement of the $\beta$-subunit in many aspects of DNA transactions other than DNA replication has been suggested.

Pol III*, the largest subassembly of pol III HE, consists of two pol III cores, two molecules of $\tau$-subunit, and one molecule each of the $\gamma, \delta, \delta', \chi$, and $\psi$ subunits. The architecture of pol III* is semi-symmetrical. As in pol III′, the $\tau$-dimer connects two pol III cores in a symmetrical configuration. On the other hand, the other five auxiliary subunits impose an asymmetry on the dimeric polymerases. Among these auxiliary subunits, $\gamma, \delta$, and $\delta'$ form a pentameric circular complex (the DnaX complex) with the $\tau$-dimer, while the $\chi$ and $\psi$ subunits form another complex that bridges the DnaX-complex and SSB (single-stranded DNA-binding protein).

### DnaX Complex

The biochemical function of the DnaX complex is to load the $\beta$-clamp on DNA. The $\delta$-subunit directly interacts with the $\beta$-subunit and opens the $\beta$-ring. Other subunits assist and regulate the action of the $\delta$-subunit. The $\tau$- and $\gamma$-subunits are encoded by the *dnaX* gene and, thus, are called DnaX proteins. $\tau$ is the full-length translation product, while $\gamma$ is a truncated protein that arises by translational frameshifting. Hence, the N-terminal portion of $\tau$ is identical to that of the $\gamma$-subunit. Furthermore, all the subunits of the DnaX complex are structurally related to the $AAA^+$ ATPase family, although only $\tau$ and $\gamma$ show ATPase activity.

ATP-binding and hydrolysis of ATP are crucial events for the clamp-loading action of the DnaX complex. In the absence of ATP, the five-subunit circular complex is in a tightly closed configuration, and inert for loading the $\beta$-subunit. In particular, the $\delta'$-subunit blocks the $\delta$-subunit's interaction with the $\beta$-subunit. When all three DnaX proteins in the complex bind ATP, the DnaX complex attains a more relaxed form in which the $\delta$-subunit readily gains access to the $\beta$-subunit. Upon association with the $\delta$-subunit, the $\beta$-ring is opened and mounted on primer-template DNA. Contact between the $\beta$–DnaX-complex and DNA activates hydrolysis of ATP bound to the DnaX-complex, which leads to dissociation of $\beta$ from the $\delta$-subunit, leaving the closed $\beta$-ring on the primer-template DNA.

### Pol III HE in Other Bacteria

Genes encoding the subunits of pol III HE have been identified in the genomes of many species of eubacteria. Among these genes, those encoding the $\alpha, \beta, \tau, \delta$, and $\delta'$-subunits are well conserved, while those for other subunits seem to be significantly divergent or lost. In many bacterial species, the *dnaX* gene does not show an obvious frameshifting signal sequence and, therefore, likely produces only the $\tau$-subunit as a single DnaX protein. A group of bacterial species (gram-positive, G + C low) including *B. subtilis*, *Staphylococcus aureus*, and *Streptococcus pyogenes* possess two distinct type-C DNA polymerases, both of which are required for chromosomal replication. Despite the apparent diversity among different species, analyses of pol III HE in bacterial species that are evolutionarily distant from *E. coli* have demonstrated that the basic architecture and biochemical functions of pol III HE are common throughout the eubacteria.

## Concurrent DNA Synthesis of Leading and Lagging Strands

*In vitro* reconstitution of the replisome with purified pol III*, $\beta$-subunit, DnaB helicase, DnaG primase, and SSB has indicated that a single pol III HE particle can simultaneously synthesize both leading and lagging strands at a replication fork. Pol III HE is a functionally and structurally asymmetric complex with twin polymerases, one of which participates in leading-strand synthesis and the other in lagging-strand synthesis. The leading-strand polymerase remains continuously

clamped to DNA, but the lagging-strand polymerase is repeatedly clamped and unclamped from DNA, to cycle from one Okazaki fragment to the next. Since the lagging-strand polymerase is held at the replication fork via the $\tau$-subunit bridge and the leading-strand polymerase, retargeting of the lagging-strand polymerase to the next primer is so efficient and rapid that the overall rate of lagging strand synthesis matches the rate of leading strand synthesis (Figure 3).

Pol III HE is initially loaded on the chromosome DNA after the first RNA primer is synthesized within or near the replication origin. One polymerase clamped on the initial RNA primer becomes the leading polymerase, and the other polymerase in the same HE complex serves

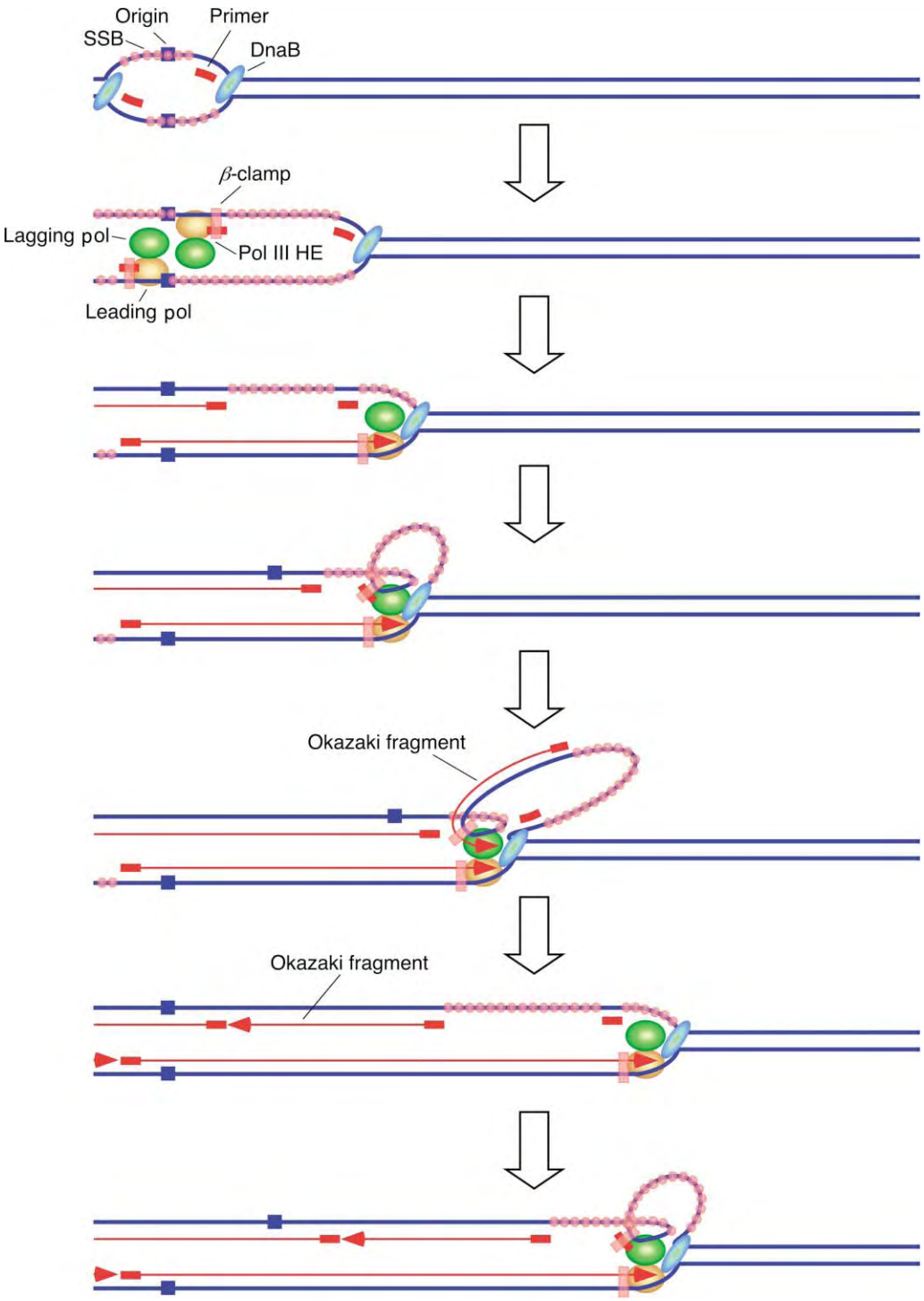



**FIGURE 3** Concurrent DNA synthesis of leading and lagging strands by the bacterial replisome.

as the lagging polymerase. An asymmetric arrangement of the DnaX complex in pol III HE is the basis for the functional asymmetry of the replicative polymerase. It has been hypothesized that the position of the DnaX complex is biased to the lagging polymerase, which limits its clamp-loading activity exclusively to the lagging strand.

## Other Biological Functions

Almost all plasmids, and the majority of bacteriophages, use pol III HE for their DNA replication. Pol III HE is involved in several kinds of DNA transactions that require synthesis of relatively long segments of DNA. These include long-patch nucleotide excision repair, *mutS*-dependent mismatch repair, homologous recombination, and replicative translocation of transposons.

### See Also the Following Articles

DNA Helicases: Dimeric Enzyme Action • DNA Helicases: Hexameric Enzyme Action • DNA Mismatch Repair and the DNA Damage Response • DNA Mismatch Repair in Bacteria • DNA Polymerase I, Bacterial • DNA Polymerase II, Bacterial • DNA Topoisomerases: Type III–RecQ Helicase Systems • recQ DNA Helicase Family in Genetic Stability

### Glossary

**AAA$^+$ ATPase family** An extension of the AAA (for ATPases associated with a variety of cellular activity) family, originally defined to include proteins with a common ~200 residue ATPase core.

**mismatch repair** A class of DNA repair that corrects mispairs and small bulge structures in DNA, which are caused mainly by replication errors.

**mutator** A phenotype showing an increased rate of spontaneous mutation, which is caused by a mutation within one of the genes for maintenance of replicational fidelity or repair of spontaneous damage to DNA and substrate nucleotides.

**nucleotide excision repair** A class of DNA repair by which a segment of DNA containing lesions is excised and replaced with resynthesized DNA.

**translesion DNA synthesis** A cellular process involving specialized DNA polymerases that counteract the replication-blocking damage to DNA.

### Further Reading

Kelman, Z., and O'Donnell, M. (1995). DNA polymerase III holoenzyme: Structure and function of a chromosomal replicating machine. *Annu. Rev. Biochem.* **64**, 171–200.

Kornberg, A., and Baker, T. (1992). *DNA Replication,* 2nd edition. Freeman, New York.

Marians, K. J. (1992). Prokaryotic DNA replication. *Annu. Rev. Biochem.* **61**, 673–719.

McHenry, C. S. (1988). DNA polymerase III holoenzyme of *Escherichia coli*. *Annu. Rev. Biochem.* **57**, 519–550.

### Biography

Dr. Hisaji Maki is a Professor in the Department of Molecular Biology at the Nara Institute of Science and Technology in Ikoma, Japan. His principal research interests are in maintenance of genetic information, spontaneous mutagenesis, and chromosome aberration. He holds a Ph.D. from Kyushu University and received his postdoctoral training in Professor Arthur Kornberg's laboratory at Stanford University. He has isolated and analyzed several mutator genes in *E. coli*, including *dnaE* and *dnaQ* which encode the $\alpha$- and $\varepsilon$-subunits of pol III holoenzyme, respectively.

# DNA Polymerases: Kinetics and Mechanism

**Kenneth A. Johnson**
*University of Texas, Austin, Texas, USA*

**Polymerases can replicate DNA with extraordinary speed and fidelity, copying a template strand at a rate of 300 base pairs per second and making a mistake only one time out of a million. When the polymerase does make a mistake, it stalls by slowing the incorporation of the next correct base pair on top of the mismatch, giving time for a proofreading exonuclease to remove the mismatched base. Selective removal of mismatched bases by the proofreading exonuclease contributes an additional factor of ~1000, resulting in a net fidelity of approximately one error in a billion bases copied. Not all polymerases achieve such high fidelity, however. Rather, each polymerase has evolved a fidelity that balances the biological needs for stability and adaptability. Although the fidelity varies greatly among the polymerases that have been examined in detail, the pathway by which a correct base pair is selected remains invariant. This article briefly summarizes our understanding of the kinetic, structural, and thermodynamic bases governing the fidelity of DNA replication.**

## Elementary Steps in Polymerization

The free energy difference between a correct and incorrect base pair is quite small (1–2 kcal/mole), which leads to a selectivity factor of only 5–30 in favoring the correct base pair over a mismatch. Therefore, the polymerase does not simply stitch together base pairs that form in solution; rather, the fidelity of DNA replication is largely a function of the kinetics of the reactions, involving nucleotide binding, recognition, and incorporation. The polymerase uses not only the base pair free energy but also the base pair geometry in selecting the correct base pair. The new base pair is buried at the active site of the polymerase, shielding it from water molecules, which enhances the free energy difference between correct and incorrect base pairs and facilitates rapid catalysis.

DNA polymerases achieve their extraordinary fidelity by using a two-step nucleotide-binding sequence to select the proper nucleotide for incorporation, as shown in Figure 1.

The deoxynucleoside triphosphate (dNTP) initially binds in a rapid equilibrium reaction to an "open" state of the enzyme to form the "ground state" complex (E·DNA·dNTP). A correct base pair enables a change in enzyme structure (at rate $k_2$) to a "closed" form in which the active catalytic residues are brought in to the proper orientation necessary for catalysis of the chemical reaction (at rate $k_3$). This two-step nucleotide-binding sequence is important for two reasons. First, the binding of the nucleotide to the open enzyme form allows a rapid selection of the correct base pair from the four competing nucleotides in solution, using base pair free energy to favor the correct base. Second, the conformational change in the enzyme to the closed form provides additional selectivity dependent upon the proper base pair geometry, and it leads to a close alignment of protein residues around the reactants, shielding them from solvent and bringing about rapid catalysis. A mismatched base pair is discriminated against at each step in the sequence. An incorrect base binds weaker in the ground state, inhibits the rate of the conformational change, and may lead to a misalignment of the reactive groups necessary for catalysis, leading to a much slower rate of incorporation.

### Selectivity Contributions of Each Step

Fidelity varies greatly for different DNA polymerases. The DNA polymerase responsible for replicating the viral genome of HIV (human immunodeficiency virus), known as reverse transcriptase (RT) because it copies RNA templates as well as DNA, has a much lower fidelity, making one mistake in only 10,000 base pairs. This higher error rate is essential for the survival of HIV by affording a fast mutation frequency that gives the virus the ability to evade the immune system and all modern drugs, by constantly changing and evolving resistance via the process of natural selection.

 © 2004, Elsevier Inc. All Rights Reserved. 

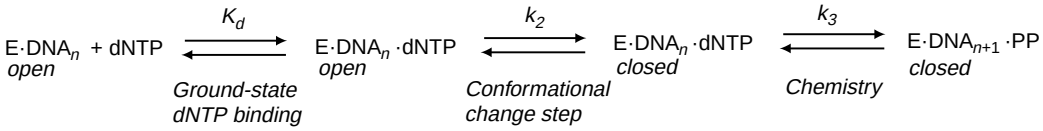



**FIGURE 1** Pathway of nucleotide binding and incorporation.

In contrast, the human DNA polymerase responsible for replicating the mitochondrial genome has an intermediate fidelity, making one error in 300,000; with proofreading, the error frequency is increased to one error in 1.5 million.

Table I shows the net selectivity observed in each step of the reaction for several polymerases. It is interesting to note that each of the polymerases shows a nearly constant selectivity of approximately 200–300 for the ground state nucleotide binding, whereas the selectivity afforded by the conformational change step (and ensuing chemistry step) contributes a widely varying selectivity. For a high-fidelity enzyme such as T7 DNA polymerase, the selectivity in the second step is 5000, leading to a net fidelity of 1.5 million. In contrast, the HIV RT shows a selectivity factor of only 50 in the conformational change step, leading to an average overall fidelity of only 13,000. Effectively, HIV RT makes at least one error each time it replicates the 9 kb viral genome, allowing the virus to rapidly evolve to evade the host immune system and any single antiviral drug. Its low fidelity is due to the small contribution of the conformational change and/or chemistry toward the net fidelity. Moreover, because HIV RT lacks a proofreading exonuclease, once a mismatch is incorporated it is not readily removed.

## Measurement of the Kinetics of Incorporation

Measurement of the kinetics of DNA polymerization is complicated by the processivity of the enzyme, defined by the tendency of the polymerase to remain bound to the DNA template/primer and to continue multiple rounds of polymerization (Table II). The processivity is calculated by the rate of polymerization divided by the rate of dissociation of the E·DNA complex and is equal to the average number of bases incorporated before the complex dissociates. Although polymerization can be quite fast (50–300 $s^{-1}$), the rate of dissociation is slow (typically 0.02–0.2 $s^{-1}$). Therefore, in the measurement of the kinetics of incorporation of a single nucleotide in the steady state with an excess of DNA and limiting enzyme, the rate that is measured is due solely to the dissociation reaction and provides no information pertaining to the nucleotide incorporation reaction, unless incorporation is slower than DNA dissociation. Therefore, steady-state rate measurements are virtually meaningless. To circumvent these difficulties, the kinetics of incorporation are measured by examining the rate of extension using single-turnover kinetic methods. A stoichiometric E·DNA complex is rapidly mixed with the correct dNTP and $Mg^{2+}$ and then quenched by mixing with EDTA to chelate metal ions needed for catalysis. The time course of extension of the DNA by one base pair is then quantified after resolution on a DNA sequencing gel.

To resolve the time dependence of extension, the reaction must be examined on the time scale of a single turnover, which usually is in the range of milliseconds. Definitive experiments require a rapid mixing device to achieve rapid mixing of small volumes. Compared to experiments using steady-state kinetic methods, single-turnover kinetic experiments may be slightly more difficult to perform, but they can be interpreted directly and often unambiguously. The nucleotide concentration dependence of the rate of the incorporation in the single-turnover reaction defines the maximum rate of incorporation, $k_{pol}$, which may be limited by $k_2$, $k_3$, or a combination of the two steps (Figure 1) and the ground state nucleotide dissociation constant, $K_d$. Because the nucleotide binding is a rapid equilibrium reaction and $k_{pol}$ is the single, rate-limiting step in polymerization, the ratio of $k_{pol}/K_d$ is equal to $k_{cat}/K_m$, the specificity constant for the reaction. Thus, this one experiment defines the key kinetic parameter governing nucleotide selectivity and resolves it into the two steps contributing to fidelity, the ground state nucleotide dissociation constant and the rate of incorporation, $k_{pol}$. Most importantly, these methods provide a measurement of

**TABLE I**

**Fidelity Contributions of Ground State Binding and Polymerization**[a]

| Polymerase | $K_{d,inc}/K_{d,cor}$ | $k_{pol,cor}/k_{pol,inc}$ | Overall fidelity |
|---|---|---|---|
| T7 DNA polymerase | 300 | 5,000 | 1,500,000 |
| Pol γ DNA polymerase | 200 | 1,500 | 300,000 |
| Klenow | 170 | 2,300 | 210,100 |
| Pol β | 290 | 610 | 19,700 |
| HIV RT | 260 | 50 | 13,000 |

[a] The average contribution of ground state binding ($K_{d,inc}/K_{d,cor}$) and the maximum rate of polymerization ($k_{pol,cor}/k_{pol,inc}$) to the net fidelity are shown for various polymerases, where *inc* represents an incorrect while *cor* represents a correct nucleotide, respectively.

**TABLE II**

**Kinetics of Polymerization**

| Polymerase | $K_d$ (nM) | $k_{pol}$ ($s^{-1}$) | $k_{pol}/K_d$ ($\mu M^{-1}s^{-1}$) | $k_{off}$ ($s^{-1}$) | Processivity | $K_{d,DNA}$ (nM) |
|---|---|---|---|---|---|---|
| T7 DNA polymerase | 18 | 300 | 15 | 0.2 | 1500 | 18 |
| Pol γ DNA polymerase | 0.8 | 40 | 50 | 0.02 | 2250 | 10 |
| Klenow | 5 | 50 | 5 | 0.2 | 250 | 5 |
| Pol β | 10 | 10 | 1 | 0.3 | 30 | 50 |
| HIV RT | 4 | 30 | 8 | 0.2 | 150 | 5 |

the equilibrium constant for the binding of the substrate to the enzyme in a complex that is poised for catalysis.

## Structural Determinants of Fidelity

The structure of T7 DNA polymerase in the closed complex is shown in Figure 2. The E·DNA·dNTP closed complex was formed with the correct dNTP and a dideoxy-terminated DNA primer/template to prevent the chemical reaction from occurring. It shows the tight arrangement of the protein around the DNA and the dNTP poised for the chemical reaction to proceed. The DNA lies largely on the surface of the protein, making contacts through the phosphodiester backbone. As the DNA approaches the active site, the structure changes from standard B-DNA to an A-form DNA with a more open minor groove that allows contacts with several residues that are thought to be important for sensing mismatches in the incoming base pair as well in the primer/template. Also important to note is that the next residue in the template strand is rotated out from the active site at 90°, and protein residues stack with both the templating base and the incoming dNTP. These contacts are all thought to be important in enforcing the proper base pair geometry.

Comparison of the T7 DNA polymerase closed complex with the structures of other polymerases in

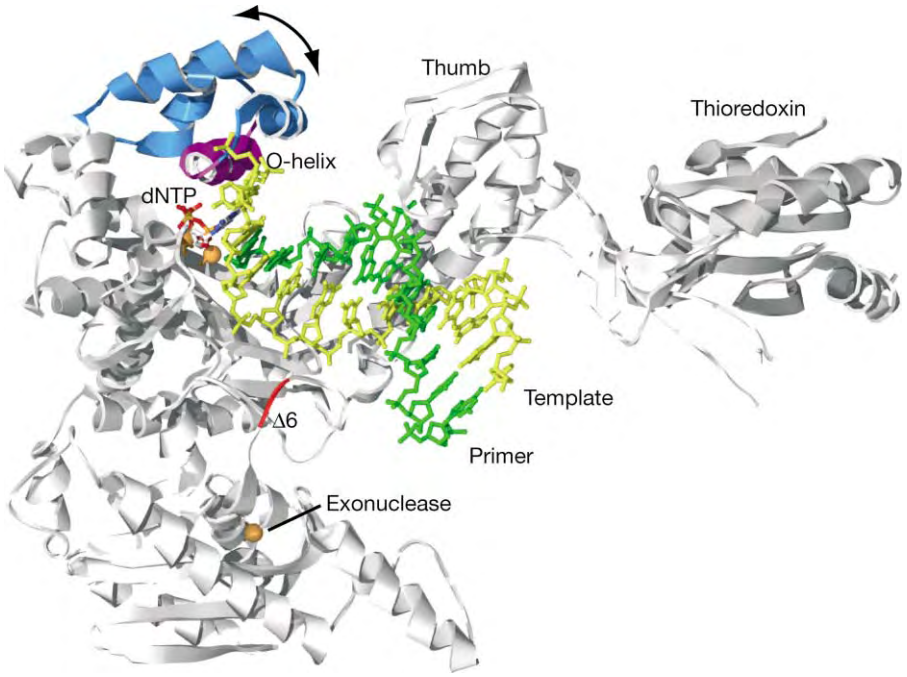



**FIGURE 2** Structure of T7 DNA polymerase. The structure shows the "closed" complex resulting from the rotation (arrow) of the recognition domain (blue) to bring the O-helix (magenta) into contact with the incoming dNTP (CPK colors). The template strand is shown in yellow, the primer strand in green, and the thioredoxin accessory protein in gray. The two metal ions at the polymerase active site and the single metal ion at the exonuclease site are shown in gold. The location of the six-residue deletion (Δ6) to create the exo-mutant is shown in red. Drawn from Protein Data Bank structure file 1T7P.

the E·DNA state reveals that there is a large conformational change in a nucleotide recognition domain involving a 45° rotation to bring key residues into contact with the dNTP at the active site. Included among these residues are positively charged lysine and arginine, amino acids that bind to the $\beta$- and $\gamma$-phosphates to facilitate catalysis, and a tyrosine that stacks with the dNTP to help align the reaction center. This conformational change step provides the most significant contribution to nucleotide selectivity by sensing the proper base pair geometry and leading to fast catalysis of the chemical reaction.

## Chemistry of Catalysis

A close-up view of the reaction center is shown in Figure 3. Two tetrahedrally coordinated $Mg^{2+}$ ions are ligated by conserved acidic residues at the active site (D475 and D654), orient the reactants, and facilitate catalysis. Metal A activates the 3′ OH and is ligated to the non-bridging oxygen of the α-phosphate of the incoming dNTP, while metal B is ligated to non-bridging oxygens of $\alpha$-, $\beta$-, and $\gamma$-phosphates and stabilizes the pyrophosphate leaving group as the reaction proceeds.

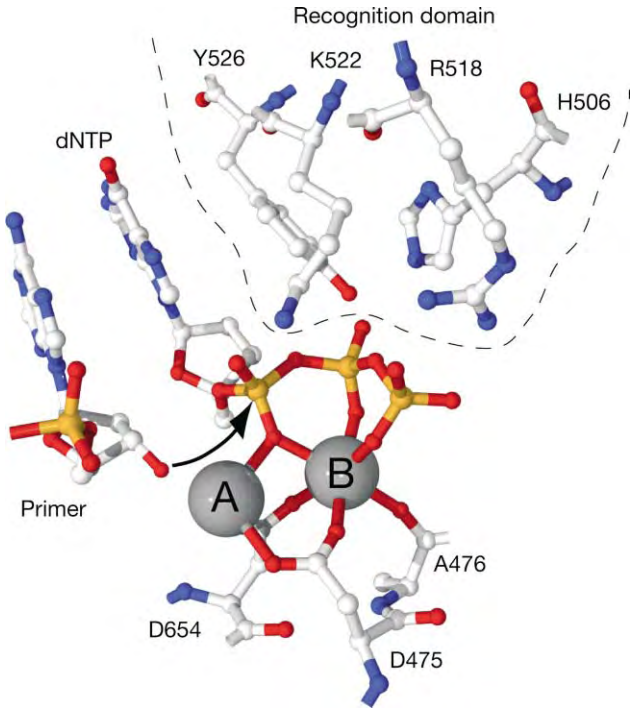



**FIGURE 3** Two-metal ion mechanism. A close-up view of the active site shows the 3′ OH of the terminal base in position to react with the incoming dNTP, bound to the two metal ions. Residues from the recognition domain contact the incoming dNTP to facilitate catalysis. Drawn from Protein Data Bank structure file 1T7P.

This two-metal ion mechanism appears to be common for all polymerases.

Catalysis is also critically dependent upon contacts made involving residues from the recognition domain. Positively charged residues K522, H506, and R518 contact the $\alpha$-, $\beta$-, and $\gamma$-phosphates, respectively; and Y526 stacks against the incoming dNTP. These contacts are formed following the conformational change and facilitate catalysis by binding and orienting the dNTP for reaction and stabilizing the development of negative charge on the pyrophosphate. Thus, the structure provides a rationale for the importance of the conformational change in nucleotide selectivity and incorporation efficiency.

## Selectivity of the Proofreading Exonuclease

The polymerase contains a proofreading function to efficiently remove mismatches after they are formed. This is accomplished by the polymerase recognizing it has made an error by stalling in its attempt to insert the next correct base on top of the mismatch to give time for the primer strand to flip over into the exonuclease site to have the 3′-terminal base excised. Although the exonuclease active site is 25 Å away from the polymerase site (see Figure 2), the DNA primer strand is able to flip back and forth between the two sites.

The selectivity of the exonuclease is governed by kinetic partitioning between the polymerase and exonuclease active sites, with the decision to excise being made at the polymerase site. As summarized in Figure 4, during successive correct nucleotide incorporation reactions, the polymerase continues down the DNA template, inserting bases at a rate of $300\ s^{-1}$. Occasionally, the DNA will dissociate from the polymerase or flip into the exonuclease site at a rate of $0.2\ s^{-1}$. Therefore, the cost of having a proofreading exonuclease site next to the polymerase active site is $0.2/(300 + 0.2) = 0.07\%$ of the correct nucleotides removed after correct incorporation. However, after the polymerase inserts a mismatched dNTP (occurring at an infrequent rate of $0.002\ s^{-1}$), the rate of incorporation of the next correct base on top of the mismatch is reduced to $0.012\ s^{-1}$, while the rate at which the primer flips over into the exonuclease site is increased to $2.3\ s^{-1}$. The probability of removing the mismatch increases to $2.3/(2.3 + 0.012) = 99.5\%$. Thus, the kinetic partitioning defining the relative probability of reaction at the polymerase and exonuclease sites is inverted when the polymerase encounters a mismatch in the primer/template.

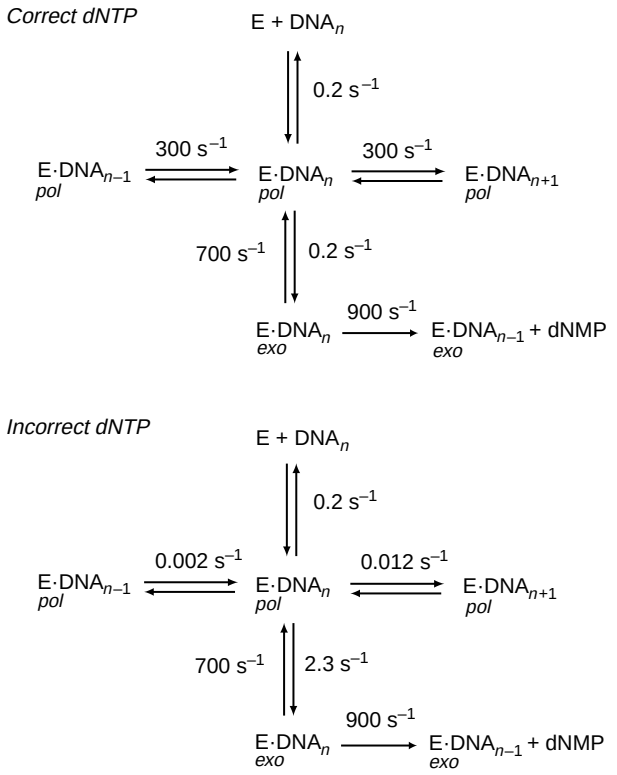



**FIGURE 4** Kinetic partitioning for exonuclease proofreading. The changes in the rates of polymerization and exonuclease removal for correct and incorrect base pairs are summarized. Correct base pairs are incorporated sequentially at a rate of 300 $s^{-1}$, while occasionally the DNA will dissociate (0.2 $s^{-1}$) or slide into the exonuclease site (0.2 $s^{-1}$). Mismatches are incorporated at a slow rate (0.002 $s^{-1}$) and extension to incorporate the next correct base is also slow (0.012 $s^{-1}$), but the rate of sliding into the exonuclease site is increased to 2.3 $s^{-1}$. This change in kinetic partitioning leads to selective removal of mismatches based upon testing for proper base pairing at the polymerase site.

The dynamics of the reaction provides a perfect solution to the problem of designing a proofreading function. A single-stranded primer in the exonuclease site is needed to excise the 3′-terminal base, but the DNA must be in duplex form in order to know whether the base pair is correct. According to the kinetics, the DNA spends most of the time at the polymerase site and makes only brief excursions to the exonuclease site, by melting out a single-stranded segment of the primer strand sufficient to reach the exonuclease site 25Å away. The decision as to whether to remove a base pair is made solely at the polymerase site, based upon the ability of the enzyme to sense a mismatch in the primer template and inhibit the rate of forward reaction, thereby changing the probability of excision versus extension.

It is interesting to note that the binding of the next correct base pair is not significantly impaired with a mismatch in the primer/template; rather, the rate of the conformational change and/or the chemical reaction is reduced by the presence of a mismatch. This again is in keeping with the postulate that one purpose of the conformational change step is to test for mismatches. The proofreading selectivity demonstrates that the polymerase is capable of sensing a mismatch not only in the incoming base pair but also at the adjacent position in the primer/template, and this ability is used to increase the selectivity at each step.

## Summary

DNA polymerases are remarkable machines central to the replication of all life forms on earth. The fidelity of different DNA polymerases varies widely in order to meet the requirements of the biology in balancing the conflicting needs for stability and adaptability. All polymerases studied in sufficient detail show a similar two-step binding mechanism to select the correct base pair using both the base pair free energy and base pair geometry.

## See Also the Following Articles

DNA Polymerase $\alpha$, Eukaryotic • DNA Polymerase $\beta$, Eukaryotic • DNA Polymerase $\delta$, Eukaryotic • DNA Polymerase $\varepsilon$, Eukaryotic • DNA Polymerase I, Bacterial • DNA Polymerase II, Bacterial • DNA Polymerase III, Bacterial • Exonucleases, Bacterial • Reverse Transcriptase and Retroviral Replication • Translesion DNA Polymerases, Eukaryotic • umuC,D Lesion Bypass DNA Polymerase V

## Glossary

**discrimination** The ratio of the specificity constant for a correct base pair divided by that for a particular mismatch.

**fidelity** The reciprocal of the error frequency; for example, an error frequency of 0.000002 is expressed as a fidelity of one error in 500,000.

**proofreading exonuclease** An exonuclease that removes a single base from the 3′ end of the primer strand.

**selectivity** The fidelity contribution of individual constants; for example, the selectivity in the ground state binding is equal to the ratio of the $K_d$ for the incorrect dNTP divided by the $K_d$ for the correct dNTP binding.

**specificity constant** The apparent second-order rate constant for nucleotide binding and incorporation, defined by the steady-state kinetic parameters, $k_{cat}/K_m$, but more accurately measured using pre-steady-state kinetic methods to define $k_{pol}/K_d$, where $k_{pol}$ is the maximum rate of incorporation and $K_d$ is the apparent ground state nucleotide dissociation constant.

## Further Reading

Doublie, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998). Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 A resolution. *Nature* **391**, 251–258.

Johnson, K. A. (1993). Conformational coupling in DNA polymerization. *Ann. Rev. Biochem.* **62**, 685–713.

Johnson, A. A., and Johnson, K. A. (2001). Fidelity of nucleotide incorporation by human mitochondrial DNA polymerase. *J. Biol. Chem.* **276**, 38090–38096.

Johnson, A. A., Ray, A. S., Hanes, J., Suo, Z., Colacino, J. M., Anderson, K. S., and Johnson, K. A. (2001). Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. *J. Biol. Chem.* **276**, 40847–40857.

Lee, H., Hanes, J., and Johnson, K. A. (2003). Toxicity of nucleoside analogs used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. *Biochemistry* **42**, 14711–14719.

Spence, R. A., Kati, W. M., Anderson, K. S., and Johnson, K. A. (1995). Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* **267**, 988–993.

## Biography

Kenneth A. Johnson is the Roger J. Williams Centennial Professor of Biochemistry at the University of Texas at Austin. His research focus is on the mechanistic and kinetic basis for DNA polymerase selectivity, especially as it pertains to the effectiveness and toxicity of drugs used to combat viral infections, especially HIV and HCV. He holds a Ph.D. from the University of Wisconsin and received postdoctoral training at the University of Chicago. He is President and CEO of KinTek Corporation, a company he founded to manufacture instruments that he designed to examine enzyme reaction kinetics on the millisecond time scale.

# DNA Replication Fork, Bacterial

**Nancy G. Nossal**
*National Institutes of Health, Bethesda, Maryland, USA*

**The complete duplex DNA chromosome of bacteria is replicated before each cell division. In *E. coli* the length of the single circular chromosome is $4.6 \times 10^6$ base pairs, and it can be copied in 40 min in rich media. Replication of bacterial DNA is thus rapid and is also extremely accurate. The error frequency during *E. coli* replication is estimated to be about $10^{-7}$, before additional correction by the mismatch-repair system. The timing of replication must be coordinated with the cell division cycle, and the synthesis of the two strands of the DNA duplex must be coordinated with each other. Replication in *E. coli* is initiated when the dnaA protein binds to a specific DNA sequence, the *oriC* origin, beginning a series of reactions in which the DNA duplex is opened to allow the binding of the other replication proteins. Replication from the origin is bidirectional, so that two replication forks moving in opposite directions are established. DNA synthesis at each fork is accomplished by a complex of many proteins, called the replisome. It includes the leading- and lagging-strand DNA polymerases, and their associated polymerase clamps and clamp loader, collectively known as the polymerase holoenzyme, which synthesize the new DNA on each strand. The replisome also contains a primosome with a primase to make short RNA chains to initiate new DNA fragments, a helicase to unwind the parental duplex, and a helicase loading protein.**

## The Two Strands of the DNA Duplex are Copied by Different Mechanisms

The two strands of a DNA duplex have opposite polarity, one goes 5′–3′ and the other 3′–5′ (Figure 1). All known DNA polymerases add new nucleotides only to the 3′ end of the chain. The parent strand that runs 3′–5′ is called the leading-strand template and serves as a template for the continuous synthesis of the new leading strand, which grows in the 5′–3′ direction. The lagging-strand template runs 5′–3′, so that the polymerase copying this strand moves away from the fork. However, microscopic studies have shown that the most recently replicated DNA on each strand is located close to the fork. On the lagging strand this is accomplished by a discontinuous mechanism of replication in which the polymerase makes short fragments that begin near the fork and extend for 1000–3000 bases (Figure 1). These fragments, which are often called Okazaki fragments in honor of their discoverer, are subsequently joined to each other by DNA ligase. Since DNA polymerases cannot begin new chains, each fragment is started by a short RNA chain (primer) that is made by a specialized RNA polymerase called a primase. To allow synthesis on the leading and lagging strands to be coordinated, Bruce Alberts proposed that the lagging strand folds into a loop, bringing the two polymerases together (Figure 2). This has been called a trombone replication model, because the expansion of the loop as each fragment is synthesized resembles a trombone slide.

## Proteins Required for DNA Replication

Much of our current understanding of bacterial replication comes from the characterization of the replication system of *E. coli*, and the simpler but mechanistically similar systems of the *E. coli* bacteriophage T7 and T4. The classes of proteins needed at the fork are shown in Figure 2, and the specific proteins in these three replication systems are shown in Table I.

### DNA Polymerase Holoenzymes

The distinguishing feature of a replicative DNA polymerase is that it is highly processive, which means that it can incorporate thousands of nucleotides each time it binds to the template. *E. coli* polymerase III and T4 polymerase remain bound because they are connected to circular-clamp proteins that surround the duplex and move with the polymerase. These clamp proteins are in turn loaded on the DNA by multisubunit complexes of proteins called clamp loaders. *E. coli* Pol III holoenzyme can be isolated as a complex containing a leading and a lagging DNA polymerase core, two clamps, and a single multi-subunit clamp loader. The clamp loader ($\gamma$-complex) is physically attached to each polymerase by the C-terminal domain of the one of two $\tau$-subunits in

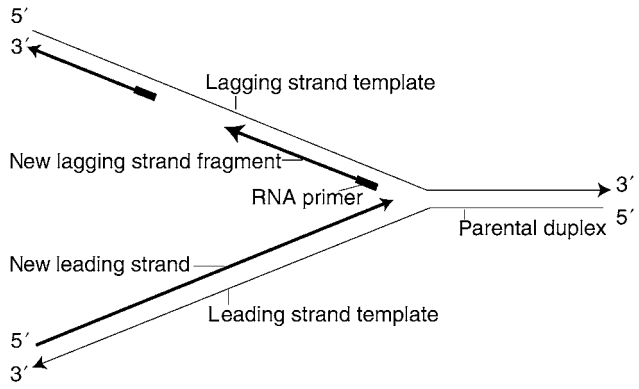



**FIGURE 1** Synthesis of the leading- and lagging-strands at a DNA replication fork. Because the two strands in a DNA duplex are antiparallel (run in opposite directions), and strands grow by addition of nucleotides to the 3′ end, the lagging strand is made by the synthesis of short fragments that are then joined together. Light lines: parental DNA template; heavy lines: new DNA.

the complex, and travels with the polymerases. The T4 proteins are less tightly connected, and are isolated as separate proteins. There is evidence that the two T4 polymerases at the fork do interact with each other at least transitorily. T7 DNA polymerase is isolated as a complex with *E. coli* thioredoxin, which increases the processivity of the polymerase.

## Primosomes

In the bacterial replication systems there is a close physical and functional relationship between the primase that makes the very short RNA chains (4–12 b) initiating each lagging-strand fragment and the helicase that unwinds the duplex ahead of the leading-strand polymerase. The length and sequences of the primers are different in each of these systems. The bacterial replicative helicases are hexamers that surround the lagging-strand template strand, and move 5′–3′ on that strand to unwind the duplex by a reaction that requires hydrolysis of nucleotide triphosphates. The loading of the *E. coli* dnaB helicase is promoted by dnaC protein, which is thought to open the preformed helicase hexamer to allow ssDNA to bind inside. The T4 59 helicase loading protein binds to the replication fork, and facilitates the assembly of the 41 helicase subunits into a hexamer surrounding the lagging-strand template. The T7 helicase and primase activities are both catalyzed by the gene 4 protein, a hexamer with adjoining rings of the primase and helicase domains of the protein.

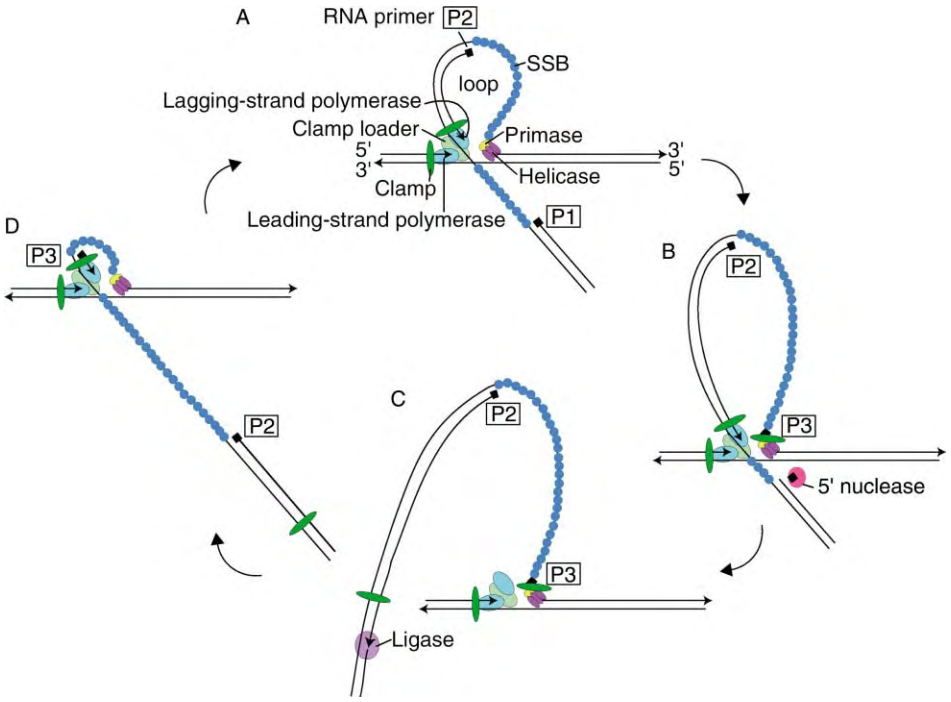



**FIGURE 2** Cycle of the reactions on the lagging strand of a bacterial DNA replication fork. The functions of the proteins are described in the text. In this model, the lagging strand has been folded to bring the leading- and lagging-strand polymerases together. (A) Extension of a lagging-strand fragment that began with primer P2 in the early elongation stage. (B) Late elongation-synthesis of a new primer (P3) for the next fragment, and hydrolysis of the primer (P1) from the previous fragment, occur as the nascent fragment is extended. (C) Fragment termination-polymerase is released after it completes the fragment. The clamp is frequently left behind. The nick between fragments is sealed by DNA ligase. (D) Polymerase is transferred to the new primer (P3) to begin elongation of the next fragment. This figure shows the reactions that must be completed in each cycle, but the order of some of the reactions, such as ligation of the adjacent fragments relative to polymerase transfer, has not been established.

**TABLE I**

**Proteins in the *E. coli* and Bacteriophage T4 and T7 DNA Replication Systems**

| Replication system | *E. coli* | Phage T4 | Phage T7 |
|---|---|---|---|
| Polymerase holoenzyme | Pol III holoenzyme | | |
| Polymerase | Polymerase core ($\alpha$, $\varepsilon$, $\theta$) | T4 DNA polymerase (gp43)[a] | T7 DNA polymerase (T7 gp5 + *E. coli* thioredoxin) |
| Clamp | $\beta$ (dimer) | gp45 (trimer) | |
| Clamp loader | $\gamma$-complex ($\gamma$, $\tau$, $\tau$, $\delta$, $\delta'$) | gp44/62 complex | |
| Primosome | | | |
| Primase | dna G | gp61 primase | gp 4[b] (hexamer) |
| Helicase | dna B (hexamer) | gp41 helicase (hexamer) | gp 4[b] (hexamer) |
| Helicase loader | dna C (hexamer) | gp59 helicase loader | |
| ssDNA-binding protein | SSB | gp 32 | gp 2.5 |
| 5′ nuclease | DNA pol I 5′ nuclease | T4 RNaseH | T7 exo6 |
| DNA ligase | *E. coli* DNA ligase | T4 DNA ligase | T7 DNA ligase |

[a] gp stands for gene product. The gene name is 43.
[b] T7 gp4 hexamer catalyzes both primase and helicase reactions.

Kinetic studies of the T7 primase–helicase show that DNA binds first to the primase site on the outside of the ring, before the ring opens to allow the DNA to reach the helicase active site in the center.

### Single-Stranded DNA-Binding Proteins

The single-stranded DNA (ssDNA) regions of the lagging strand are covered by tight single-stranded DNA-binding proteins called SSB = s. In addition to protecting the ssDNA from nucleases, SSB proteins bind specifically to several other replication proteins, including polymerases, primases, and in the T4 system, the helicase loading protein, and modulate their activities.

### 5′–3′ Nucleases and DNA Ligases

The RNA primers on lagging-strand fragments must be removed before the fragments can be joined by DNA ligase. The enzymes responsible for removing the primers are 5′–3′ nucleases that are capable of degrading both RNA:DNA and DNA:DNA duplexes. Thus, they can remove the RNA primers and a short stretch of adjoining DNA from the discontinuous fragments.

## DNA Replication Cycle

### Leading-Strand Synthesis

The leading strand at the replication fork is synthesized continuously (Figure 2), beginning with an RNA primer that is made, in different systems, by either an RNA polymerase or a primase. Once the leading-strand polymerase and helicase are loaded, this polymerase can in principle remain bound to complete synthesis of the chromosome. The rate of synthesis by the polymerase, and duplex unwinding by the helicase, are much greater when these proteins work together at a replication fork, than when these reactions occur separately. The $\tau$-subunit of the *E. coli* clamp loader connects the polymerase holoenzyme with the helicase. Although synthesis on the leading strand is extremely processive, recent evidence shows that bacterial replication forks frequently stall before completing the chromosome and need to be reassembled.

### Lagging-Strand Synthesis

Because the lagging strand is made by joining short fragments, there is a cycle of reactions needed to initiate, elongate, and seal these fragments (Figure 2A–D). These reactions must be coordinated with each other, as well as with the reactions on the leading strand. The lagging-strand cycles are completed in only a few seconds because the fragments are 1000–3000 bases, and replication is proceeding at 400–1000 bases/second.

#### Early Elongation Stage

In the beginning of the elongation stage of this cycle, the clamped lagging strand polymerase is synthesizing a fragment that began with the primer labeled P2 (Figure 2A). At the same time, the helicase surrounding the lagging-strand template at the fork is unwinding the duplex ahead of the leading-strand polymerase.

SSB proteins coat the ssDNA between the helicase and the nascent elongating fragment, and the ssDNA between the lagging-strand polymerase and the previous fragment. Although the protein-covered ssDNA in Figure 2 is shown as a linear array for simplicity, there is evidence discussed below that it is actually in a more compact structure.

### Late Elongation and Primer Synthesis

Each lagging-strand cycle can be completed rapidly because reactions required to prime the next fragment, elongate the present fragment, and remove primers from the previous fragment can occur simultaneously. In the latter part of the elongation stage (Figure 2B) as synthesis of the nascent fragment continues, primase, associated with the helicase at the fork, makes the RNA primer (P3) that will be used to start the next fragment. In the *E. coli* and T4 systems a clamp is loaded on the new primer by the clamp loader. The *E. coli* clamp loader is bound to the two polymerases at the fork, as well as to the helicase, while the clamp is recruited from solution. Dilution experiments suggest that both the T4 clamp and clamp loader must be provided from solution at the beginning of each cycle. At the same time, the $5'-3'$ nuclease is removing the RNA primer (P1) and a small amount of adjacent DNA from the previous fragment. This is the DNA that was first added to the primer, and its removal may increase the accuracy of replication. In the T4 system the SSB (T4 32 protein) between the polymerase and the nuclease increases the rate of these two reactions, and controls the extent of DNA removed along with the RNA primer.

### Fragment Termination

When the lagging-strand polymerase completes the nascent fragment (Figure 2C), creating a nick, polymerase is released, but the clamp is frequently left behind on the DNA. In *E. coli*, the C-terminal domain of the $\tau$-subunit of the clamp loader acts as an unloader, releasing the lagging-strand polymerase core from the $\beta$-clamp. The T4 proteins are less tightly bound, and there is no evidence that the clamp loader is needed to disengage the clamp and polymerase. If the polymerase fails to be released at the nick, it can continue strand-displacement synthesis, forming a flap from the $5'$ end of the downstream fragment. However, all of the prokaryotic $5'$ nucleases with a role in primer removal have flap endonuclease activities, which can remove these displaced strands. The nicks that are formed between adjacent fragments are sealed by DNA ligase.

### Transfer of Polymerase to the New Primer

The *E. coli* lagging-strand polymerase released from the completed fragment remains attached to the clamp loader at the fork, and can be transferred directly to the clamped new primer (P3) to begin synthesis of the next fragment (Figure 2D). Under some conditions, the addition of primase acts as a signal that causes the lagging-strand polymerase to be released prematurely, before the previous fragment is finished. This leaves a gap between fragments that must ultimately be repaired by loading another polymerase. Structural and functional studies of the T7 primase-helicase show that the DNA-binding domain of the primase binds the new primer and transfers it to the lagging-strand polymerase.

### Coordination of Leading and Lagging-Strand Synthesis

The *E. coli* leading and lagging-strand polymerases are connected because they are each joined to the same clamp-loading complex. The two polymerases at the T4 and T7 replication fork are not joined through a clamp loader. However, on synthetic templates in which dGTP can only be incorporated into the leading

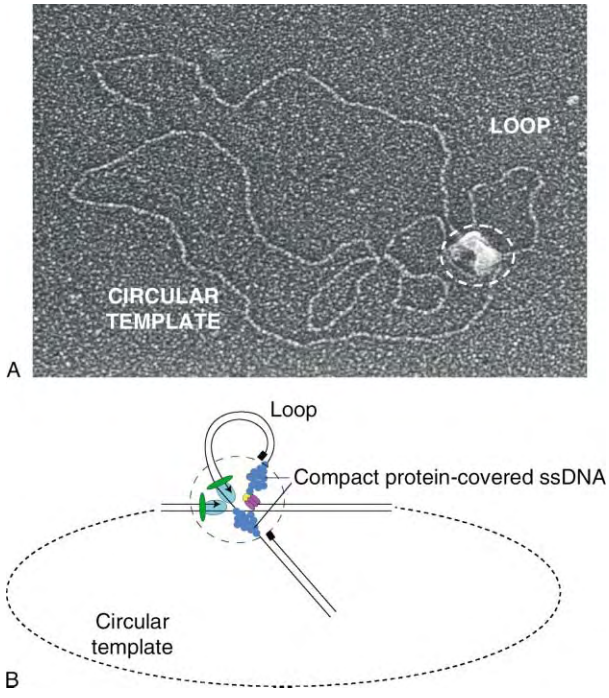



**FIGURE 3** The single-stranded DNA on the lagging template is in a compact structure. (A) Electron micrograph of a replication fork with bacteriophage T4 proteins. The loop at the fork is all duplex DNA. The dense complex at the fork contains the replication proteins, as well as protein-covered ssDNA that has been folded into a compact structure. (Adapted from Chastain *et al.* (2003) *J. Biol. Chem.* **278**, 21276). (B) Model of a fork with compact ssDNA. Compare this model with that in Figure 2A.

strand and dCTP into the lagging strand, leading-strand synthesis stopped when a chain terminator was added to block lagging-strand synthesis, indicating a close interaction between the polymerases on the two strands. In addition, dilution experiments suggest that these phage polymerases can recycle from one lagging-strand fragment to the next.

## The ssDNA on the Lagging Strand is in a Compact Structure

Electron microscopic analysis of DNA being replicated by the phage T7 or T4 proteins has confirmed that there is a loop at the replication fork, as predicted by the trombone replication model. While the model envisioned that the loops would be composed of the new duplex lagging-strand fragment and the ssDNA behind it (Figure 2A), the observed loops were entirely double stranded (Figure 3). The dense complex at the fork contains the replication proteins, as well as the protein-covered ssDNA folded into a compact structure. The path of the DNA and the proteins in this structure remain to be determined. The compact nature of the lagging-strand template may limit access of the primase and clamp loader, and thus be a factor in controlling when new lagging-strand fragments are initiated.

## Replication Factories

Studies of the location of *B. subtilis* DNA polymerase first showed that replisome proteins are concentrated into discrete regions of living cells that have been called replication factories. It is likely that the reactions at a replication fork take place as DNA template is pulled through a stationary replisome, rather than having the replisome proteins move along the DNA.

## See Also the Following Articles

DNA Helicases: Hexameric Enzyme Action • DNA Polymerase III, Bacterial • DNA Replication: Initiation in Bacteria • Processivity Clamps in DNA Replication: Clamp Loading • Sliding Clamps in DNA Replication: *E. coli* β-Clamp and PCNA Structure

## Glossary

**DNA helicase** An enzyme that unwinds the two strands of a DNA duplex.

**DNA ligase** An enzyme that covalently joins two DNA strands together.

**DNA polymerase** An enzyme that synthesizes DNA by incorporating nucleotides at the 3′ end of the new strand that are complementary to the nucleotides on the strand serving as the template.

**lagging strand** The strand at the replication fork that is synthesized discontinuously by making shorter fragments that are sealed together by DNA ligase.

**leading strand** The strand at the replication fork that is synthesized continuously as one long strand.

**primase** An enzyme that synthesizes the short RNA primers that are used to begin the discontinuous fragments on the lagging strand of a replication fork.

## Further Reading

Ahnert, P., Picha, K. M., and Patel, S. S. (2000). A ring-opening mechanism for DNA binding in the central channel of the T7 helicase-primase protein. *Embo. J.* **19**, 3418–3427.

Alberts, B. (2003). DNA replication and recombination. *Nature* **421**, 431–435.

Chastain, P., Makhov, A. M., Nossal, N. G., and Griffith, J. D. (2003). Architecture of the replication complex and DNA loops at the fork generated by the bacteriophage T4 proteins. *J. Biol. Chem.* **278**, 21276–21285.

Davey, M. J., Jeruzalmi, D., Kuriyan, J., and O'Donnell, M. (2002). Motors and switches: AAA+ machines within the replisome. *Nat. Rev. Mol. Cell Biol.* **3**, 826–835.

Kato, M., Ito, T., Wagner, G., Richardson, C. C., and Ellenberger, T. (2003). Modular architecture of the bacteriophage T7 primase couples RNA primer synthesis to DNA synthesis. *Mol. Cell* **11**, 1349–1360.

Kornberg, A., and Baker, T. (1992). *DNA Replication*, 2nd edition. W. H. Freeman, San Francisco.

Lemon, K. P., and Grossman, A. D. (1998). Localization of bacterial DNA polymerase: Evidence for a factory model of replication. *Science* **282**, 1516–1519.

Leu, F. P., Georgescu, R., and O'Donnell, M. (2003). Mechanism of the *E. coli* tau processivity switch during lagging-strand synthesis. *Mol. Cell* **11**, 315–327.

## Biography

Dr. Nancy Nossal is Head of the Laboratory of Molecular and Cellular Biology in the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institutes of Health. Her research interests are in understanding the molecular mechanisms of DNA replication, and she has worked extensively with the phage T4 replication system. She holds a Ph.D. in biological chemistry from the University of Michigan and was a postdoctoral fellow at the National Institutes of Health.

# DNA Replication Fork, Eukaryotic

**Lori M. Kelman**
*Montgomery College, Germantown, Maryland, USA*

**Jerard Hurwitz**
*Memorial Sloan-Kettering Cancer Center, New York, USA*

**Zvi Kelman**
*University of Maryland Biotechnology Institute, Rockville, Maryland, USA*

**During the S phase of the cell cycle, DNA replication duplicates chromosomes into two identical copies, which segregate to daughter cells during mitosis. In DNA replication, a double-stranded molecule of DNA is copied into two daughter molecules. DNA replication occurs at replication forks – structures consisting of DNA and replication proteins that allow the two strands of DNA to be copied accurately and completely.**

## Introduction

All organisms must replicate their chromosomal DNA in order to propagate their genetic information. During the S phase of the cell cycle, DNA replication duplicates chromosomes into two identical copies that segregate to daughter cells during mitosis. Chromosomal DNA replication begins at regions of the chromosomes called origins of replication and can be divided into three phases: initiation, elongation, and termination. In the initiation stage, an origin recognition protein (ORP) binds the origin of replication and recruits additional initiation factors to the origin. Next the helicase is recruited to the DNA to form the initial replication bubble. The single-stranded DNA (ssDNA) exposed behind the helicase is coated with ssDNA-binding protein (SSB). The polymerase and the rest of the replication machinery are associated with the SSB/origin complex to form the two replication forks and to initiate bidirectional DNA synthesis (the elongation phase). During termination, replication forks collide and are resolved, and the resulting daughter DNA molecules are completed and separated. A number of proteins and complexes that participate in replication fork progression are described below.

Due to the antiparallel nature of DNA and the unidirectionality of the polymerase, one strand of the chromosome is synthesized continuously (the leading strand) while the other is copied discontinuously (the lagging strand) as a series of Okazaki fragments (Figure 1). It is believed that at the replication fork, the two polymerases responsible for replicating the leading and lagging strand are associated (either directly or indirectly via other molecules) (Figure 1). Thus, the replication of the two strands is coupled.

## Minichromosome Maintenance (MCM) Complex

MCM is a family of six proteins (Mcm2–7, molecular masses of 101, 91, 97, 82, 93, and 81 kDa, respectively) with highly conserved amino acid sequences between the six different polypeptides. All MCM proteins are essential for cell viability and have been identified in all eukarya. In addition to forming a heterohexamer, *in vivo* and *in vitro* studies have revealed the presence of several additional MCM complexes composed of different combinations of the MCM proteins. Biochemical studies with the various complexes in yeast and mammals have shown that a dimeric complex of the Mcm4,6,7 heterotrimer contained $3'–5'$ DNA helicase activity, ssDNA binding, and DNA-dependent ATPase activities, while its interactions with either Mcm2 or Mcm3,5 inhibited the helicase activity. However, all six proteins were shown to be essential for replication fork movement. Based on genetic and biochemical studies, the MCM complex is presumed to be the helicase responsible for the separation of duplex DNA during chromosomal replication.

## Replication Protein A (RPA)

RPA (also called replication factor A, RFA) is the eukaryotic single-stranded DNA-binding protein. It is a heterotrimeric complex of proteins with molecular

 © 2004, Elsevier Inc. All Rights Reserved. 

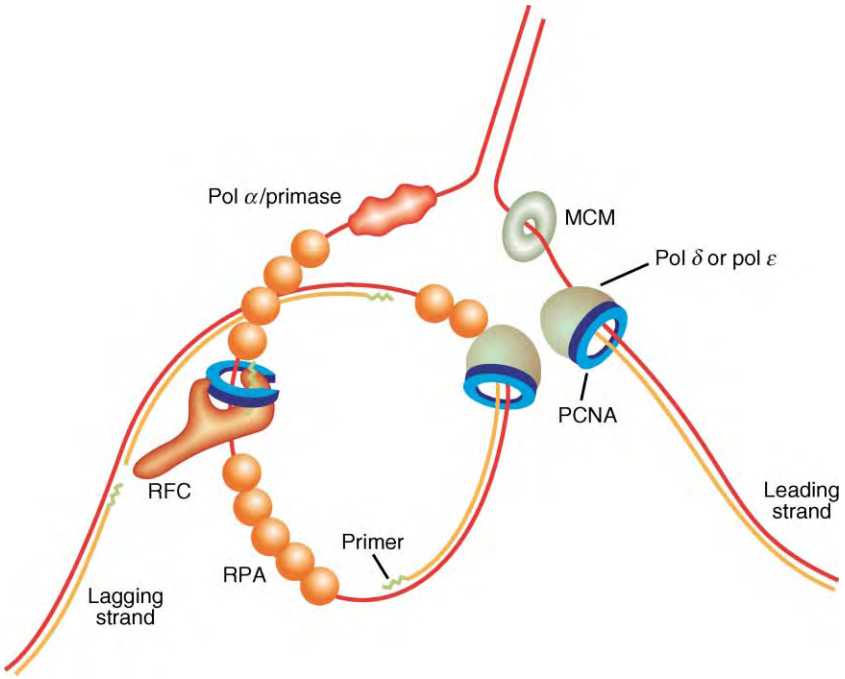



**FIGURE 1** A schematic representation of the proteins at the eukaryotic replication fork. Although it is not shown in the figure, it is thought that all proteins shown interact with each other.

masses of 14, 32, and 70 kDa; these essential proteins have been found in all eukarya studied. SSBs are essential components of all replication systems. The protein coats the ssDNA exposed behind the helicase, thus protecting it from attack by nucleases and chemical modification. In addition, RPA stimulates the activity of DNA polymerases by removing secondary structures that interfere with polymerase movement, creating a uniform substrate for polymerase activity. RPA was shown to play an instrumental role in the coordination of DNA synthesis on the lagging strand and to interact with a large number of cellular factors needed for DNA metabolism.

## DNA Polymerase α/Primase (Pol α/Primase) Complex

DNA polymerases are incapable of initiating DNA synthesis *de novo* and require DNA primases, which synthesize short RNA primers on template DNA that are subsequently extended by the polymerase. In eukarya, DNA primase is part of a four-subunit complex, the Pol α/primase complex, containing subunits with molecular masses of 180, 68, 58, and 48 kDa. Two of the subunits, p48 and p58, are required for primase activity, with p48 serving as the catalytic unit. Primase synthesizes short RNA primers (8–12 nucleotides (nt)) which are then elongated by Pol α to ~30 nt, forming pre-Okazaki fragments. These RNA–DNA hybrid molecules are recognized by the polymerase accessory complex, replication factor C (RFC) to initiate processive DNA synthesis by the replicative polymerase. On the lagging strand Pol α/primase activity is required at the initiation of each Okazaki fragment.

## Polymerase Accessory Proteins

The processivity of DNA polymerases is quite low. This means that only a few nucleotides are incorporated into the newly synthesized DNA before the polymerase dissociates from the substrate. Processivity is conferred by a ring-shaped protein, referred to as the DNA polymerase sliding clamp (also called DNA polymerase processivity factor) that encircles DNA and acts to tether the polymerase catalytic unit to the primed template for processive DNA synthesis. The eukaryotic sliding clamp is proliferating cell nuclear antigen (PCNA). However, PCNA cannot assemble itself around the DNA, but must be loaded onto DNA by a protein complex, known as the clamp loader (also called polymerase accessory complex), which in eukarya is RFC. RFC recognizes the 3′ end of the single strand/duplex (primer–template) junction of the pre-Okazaki fragment and utilizes ATP hydrolysis to assemble PCNA around the primer. PCNA encircles the primer DNA and then binds the polymerase catalytic

unit, allowing rapid and processive DNA synthesis. Upon completion of an Okazaki fragment, the polymerase dissociates, leaving the clamp assembled around the duplex DNA.

### Proliferating Cell Nuclear Antigen (PCNA)

PCNA, the eukaryotic sliding clamp, is a ring-shaped homotrimer of a 29 kDa protein. Upon its assembly around the primer of the pre-Okazaki fragment by RFC, PCNA associates with Pol $\delta$ or Pol $\varepsilon$ to initiate processive DNA synthesis. *In vitro* studies demonstrated that upon assembly around duplex DNA, PCNA could slide freely and bidirectionally along the duplex. Although PCNA has no enzymatic activity, it plays a major role in chromosomal DNA replication. Besides its role as a processivity factor for Pol $\delta$ and Pol $\varepsilon$, PCNA was shown to play a regulatory role during chromosomal replication. A number of proteins that inhibit DNA synthesis were shown to operate via interaction with PCNA, preventing its association with the polymerase. Upon completion of an Okazaki fragment, PCNA disengages from the polymerase and is left on the duplex DNA. The PCNA molecules left on replicated DNA play diverse roles in postreplication DNA metabolic processes.

### Replication Factor C (RFC)

RFC, the eukaryotic clamp loader, is a five-subunit complex of 140, 40, 38, 37, and 36 kDa proteins (for Rfc1–5, respectively). All five subunits are essential for cell viability. The amino acid sequences of each subunit reveal significant homology in seven regions (boxes II–VIII). The large subunit (Rfc1) contains an additional box (box I) within its N-terminal region. RFC recognizes the 3′ end of the pre-Okazaki fragment and utilizes ATP hydrolysis to assemble PCNA around the duplex DNA. It was shown that the interactions between RFC and RPA play a role in the switch that removes Pol $\alpha$/primase from the DNA, allowing the loading of PCNA. In addition to its role as a clamp loader, *in vitro* studies suggest that RFC also functions as a clamp unloader, i.e., removing free PCNA from DNA.

## Polymerase $\delta$ (Pol $\delta$) and Polymerase $\varepsilon$ (Pol $\varepsilon$)

Both Pol $\delta$ and Pol $\varepsilon$ are essential polymerases required for chromosomal replication. However, their precise function at the replication fork remains unclear. Although Pol $\delta$ is found in all eukarya, its polypeptide composition is different in different organisms. To date, Pol $\delta$ isolated from either mammals or *Schizosaccharomyces pombe* is a heterotetramer of 125, 66, 50, and 12 kDa protein (only the human molecular masses are given) while the *Saccharomyces cerevisiae*, Pol $\delta$, is a heterotrimer. *In vitro* studies with these enzymes indicate that all Pol $\delta$ complexes are monomers. Following the assembly of PCNA at the primer, Pol $\delta$ associates with the clamp to initiate rapid and processive DNA synthesis.

Pol $\varepsilon$ is a four-subunit complex of proteins with molecular masses of 261, 59, 17, and 12 kDa (for the mammalian complex). As is the case with Pol $\delta$, Pol $\varepsilon$ is a monomeric polymerase. Like Pol $\delta$, the PCNA clamp is required for the Pol $\varepsilon$-catalyzed DNA synthesis in the presence of physiological salt concentrations. However, at low ionic strength Pol $\varepsilon$ alone can elongate primed DNA chains, in contrast to Pol $\delta$.

Reconstitution of the simian virus 40 (SV40) DNA replication system with mammalian enzymes demonstrated that only Pol $\delta$ was required for replication of both leading and lagging strands and did not require Pol $\varepsilon$. Both *in vivo* experiments in yeast and *in vitro* experiments with the *Xenopus* cell free replication system indicate that Pol $\varepsilon$ and Pol $\delta$ are required for replication. Genetic experiments in yeast showed that Pol $\varepsilon$ is located at the replication fork. Furthermore, mutational rate studies in *S. cerevisiae* suggest that the proofreading functions of Pol $\delta$ and Pol $\varepsilon$ act on different DNA strands. However, the precise location of each polymerase (i.e., lagging or leading strand) could not be deduced. These results suggest that each polymerase acts on a specific strand during replication. However, conflicting results in yeasts indicate that cells deleted of the catalytic domain of the large subunit of Pol $\varepsilon$ (at the N terminus) are viable, provided that the C terminus of this subunit is expressed. The C terminus was shown to play an important role in the regulation of DNA replication. Interestingly, point mutations in the conserved catalytic site of Pol $\varepsilon$ result in loss of viability. Thus, although Pol $\varepsilon$ is normally present at the replication fork, in the absence of the catalytic portion of its large subunit, it is likely that Pol $\delta$ can substitute for its function. Further studies are needed to determine the cellular functions of Pol $\delta$ and Pol $\varepsilon$.

## Topoisomerases

During replication, DNA is supercoiled in front of and behind the polymerase. Positive supercoils are produced in front of the replication fork, while negative supercoils result behind it. Topoisomerases prevent excessive supercoiling and regulate the level of supercoiling within the DNA molecule. These enzymes are essential for replication fork progression.

Topoisomerases introduce transient breaks in the phosphodiester backbone of the DNA, which allow supercoils to be added or removed.

## Conclusion

The replication of eukaryotic chromosomes is a very complex and highly coordinated process involving dozens of proteins. Although most of the proteins assembled at the replication forks have been identified and isolated, the precise biochemical properties and roles of some of these proteins remain to be elucidated.

### See Also the Following Articles

DNA Polymerase $\alpha$, Eukaryotic • DNA Polymerase $\beta$, Eukaryotic • DNA Polymerase $\delta$, Eukaryotic • DNA Polymerase $\varepsilon$, Eukaryotic • DNA Polymerases: Kinetics and Mechanism • DNA Replication: Eukaryotic Origins and the Origin Recognition Complex • DNA Topoisomerases: Type I • DNA Topoisomerases: Type II • DNA Topoisomerases: Type III–RecQ Helicase Systems • Translesion DNA Polymerases, Eukaryotic

### Glossary

**bidirectional DNA replication** DNA synthesis that originates at a bidirectional origin results in the formation of two replication complexes leading to two replication forks that move in opposite directions.

**DNA polymerase** Enzyme that utilizes a primed DNA template to catalyze the synthesis of DNA.

**helicase** Enzymes that use the energy derived from hydrolysis of nucleoside triphosphates to sever the hydrogen bonds that hold each strand of duplex DNA together.

**primase** Enzyme that synthesizes small RNA chains *de novo* on ssDNA resulting in RNA–DNA hybrids which are used to prime DNA synthesis.

### Further Reading

Baker, T. A., and Bell, S. P. (1998). Polymerases and the replisome: Machines within machines. *Cell* **92**, 295–305.

Bambara, R. A., Murante, R. S., and Henricksen, L. A. (1997). Enzymes and reactions at the eukaryotic DNA replication fork. *J. Biol. Chem.* **272**, 4647–4650.

DePamphilis, M. L. (ed.) (1996). *DNA Replication in Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, New York.

Hübscher, U., and Seo, Y. S. (2001). Replication of the lagging strand: A concert of at least 23 polypeptides. *Mol. Cells* **12**, 149–157.

MacNeill, S. A., and Burgers, P. M. J. (2000). Chromosomal DNA replication in yeast: Enzymes and mechanisms. In *Frontiers in Molecular Biology: The Yeast Nucleus* (P. Fontes and J. Beggs, eds.) pp. 19–57. IRL Press, Oxford.

O'Donnell, M., Jeruzalmi, D., and Kuriyan, J. (2001). Clamp loader structure predicts the architecture of DNA polymerase III holoenzyme and RFC. *Curr. Biol.* **11**, R935–R946.

Tye, B. K. (1999). MCM proteins in DNA repelication. *Ann. Rev. Biochem.* **68**, 649–686.

Vivona, J. B., and Kelman, Z. (2003). The diverse spectrum of sliding clamp interacting proteins. *FEBS Lett.* **546**, 167–172.

Waga, S., and Stillman, B. (1998). The DNA replication fork in eukaryotic cells. *Annu. Rev. Biochem.* **67**, 721–751.

### Biography

Lori M. Kelman is a Professor of biotechnology at Montgomery College, Germantown, MD. She received an A.B. in biochemistry from Mount Holyoke College, a M.S. in biology from St. John's University, a MBA in Management from Iona College, and a Ph.D. in molecular biology from Cornell University. She is Editor of *BIOS*, a quarterly journal of biology.

Zvi Kelman is an Assistant Professor at the Center for Advanced Research in Biotechnology, one of the University of Maryland Biotechnology Institutes. His work is focused on DNA replication. He received his Ph.D. in Molecular Biology from the Cornell University School of Medicine.

Jerard Hurwitz, whose research is focused on DNA replication, is a Member of the Molecular Biology Program at Memorial Sloan-Kettering Cancer Center. He received his Ph.D. in Biochemistry from Western Reserve University and has held faculty positions at Washington University, St. Louis, New York University School of Medicine and Albert Einstein College of Medicine.

# DNA Replication, Mitochondrial

**David A. Clayton**
*Howard Hughes Medical Institute, Chevy Chase, Maryland, USA*

**Nearly all eukaryotic cells contain organelles called mitochondria that contain enzymes which utilize oxygen and nutrient molecules, such as sugars, to produce most of the ATP that in turn provides energy to support cellular molecular activities. The mitochondria, in addition to being the principal sites of energy production, contain their own genomes, mitochondrial DNA (mtDNA), which are different structurally and functionally from the much larger nuclear genome. DNA replication is the process by which new DNA is synthesized by copying the strands of pre-existing DNA. Replication of mtDNA occurs by a distinctive mechanism independent of the replication of DNA in the cell nucleus. Proper synthesis and maintenance of mtDNA and the mitochondrion as a whole requires the coordinated participation of both nuclear and mtDNA gene products.**

## Mitochondrial Constituents and their Functions

Mitochondria comprise several hundred macromolecules, including mtDNA, mitochondrial RNA (mtRNA), and proteins.

### mtDNA

Although certain cells can exist without mtDNA under special nutrient and growth conditions, where oxidative phosphorylation activity is not essential, in all other cases, mtDNA is needed. This is due to the fact that mtDNA encodes some of the proteins necessary for mitochondrial function in energy production. The sizes of mitochondrial genomes are quite broad. Vertebrate mtDNAs are typically 16–18 Kb in size, with yeast mtDNA being several times larger. Plant mtDNAs can be 30-fold larger than vertebrate mtDNA. In addition, the number and identity of mtDNA genes is not constant across all mtDNAs. Human (and vertebrate) mtDNAs contain 37 genes, of which 13 are for proteins involved in bioenergetics. Although larger mtDNAs can contain more genes, the coding information is not directly linear with genome size.

### RNA

The RNA inside the mitochondrion is almost exclusively that which is encoded by mtDNA. For mammals, this generally includes two rRNAs and 22 tRNAs. These 24 RNAs are thought to be necessary and sufficient to support fully the mitochondrial translation machinery, responsible for decoding the 13 mammalian mRNAs for mtDNA-encoded proteins.

Other RNAs, encoded by nuclear genes, include additional tRNAs, in cases where mtDNA contains an insufficient number of its own tRNA genes. In addition, certain RNA-processing activities, consisting of enzymes with nucleus-encoded RNA components, have been implicated as participants in processing mtRNA sequences. These include an RNase P for processing tRNA precursors and an RNase that cleaves mtRNA sequences at the origin of leading-strand DNA replication. This latter activity is thought to provide 3′-primer RNA ends for elongation by mtDNA polymerase.

### Protein

The protein components of mitochondria are of several classes. Although mitochondria play key roles in apoptosis, calcium regulation, iron metabolism, and the synthesis of amino acids, sterols, and heme, the principal function of the organelle is energy production. It is, therefore, not surprising that all of the mtDNA-encoded proteins participate in the formation of one or another of the several bioenergetic complexes that support the chain of reactions leading to ATP production. Although the vast majority of mitochondrial proteins are encoded by nuclear genes, those encoded by mtDNA are essential and there are no nuclear genes present that can substitute for a loss of mtDNA.

The several hundred nucleus-encoded proteins that comprise the bulk of mitochondrial proteins fall roughly into two classes. One class is devoted to the task of energy production and mitochondrial function, including maintenance and expression of mtDNA. The second population consists of structural elements that provide the infrastructure that maintains basic mitochondrial morphology, mobility, and organelle physiology.

 © 2004, Elsevier Inc. All Rights Reserved. 

Most mitochondrial proteins, being nuclear-gene products, are produced by translation on cellular ribosomes and then targeted to mitochondria by their sequence (the most common feature directing the process is a signal sequence at one end of the protein, the amino terminus). Physical importation of the protein involves specific portions of the mitochondrion's inner and outer membranes that form ports for entry of these proteins. Initially, a general internalizing import mechanism applies to most proteins regardless of their final destination within the mitochondria. This is followed by other mechanisms leading to sorting of protein according to function.

## Replication of the Mitochondrial Genome

Replication of mtDNA occurs within the confines of the mitochondrial-organelle network, which is usually organized dispersively throughout the cell's cytoplasm. As such, it represents a separately managed genome, physically and functionally distinct from nuclear DNA replication. The cellular copy number of mtDNA varies according to cell type. For example, mature lymphocytes have a few hundred mitochondrial genomes, whereas mammalian oocytes contain tens of thousands. Unlike nuclear DNA replication, which is subject to cell cycle control, it appears that mtDNA is able to initiate and complete a round of replication in dividing cells at any time.

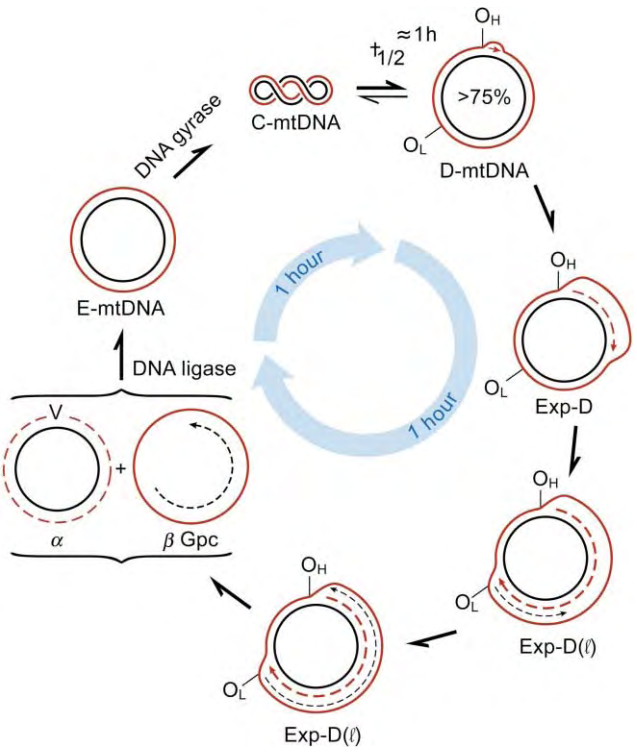



**FIGURE 1** Diagram of mtDNA replicative intermediates. In the strand-displacement model closed-circular mtDNA (C-mtDNA) is in equilibrium with D-loop mtDNA (D-mtDNA). Productive replication is defined by elongation of the leading strand (red), which progresses around the genome (dashed red; Exp-D) until exposure of the major lagging-strand origin, which sponsors initiation of synthesis in the counterclockwise direction. End products are the new $\alpha$- and $\beta$-circles, which are finished to closed circles (E-mtDNA). Superhelical turns are introduced (C-mtDNA) and finally a new D loop is synthesized. There is a rapid conversion back-and-forth between D-mtDNA and C-mtDNA.

### Mammalian mtDNA

The historic replication model posits that leading-strand replication of mammalian mtDNA begins at closely spaced, defined sites located downstream from a major transcription promoter and proceeds unidirectionally with displacement of the parental leading strand until approximately two-thirds of the closed circular mtDNA has been copied (Figure 1). As a consequence, the replication fork passes a major origin for lagging-strand synthesis, leaving it in single-stranded form. Displacement as a single strand is thought to allow the characteristic secondary structure of this origin to occur, thereby permitting initiation of lagging-strand synthesis. A natural consequence of the separate and distinct locations of the two origins is that the two segregated progeny mtDNA circles are of two types: one a duplex circle with a newly synthesized leading strand and the other a gapped circle with a partial newly synthesized lagging strand. In each case, the final steps of synthesis and closure result in the mature closed circular mtDNA products.

Convincing biochemical isolation and characterization of mammalian mtDNA began in the 1960s. The key technical breakthrough was the use of dye-salt buoyant density centrifugation to isolate even relatively rare closed-circular DNAs in highly purified form. This technique was exploited to perform mtDNA analyses by sophisticated analytical centrifugation techniques, by electron microscopy, and eventually by gel electrophoresis. It is fortuitous that mammalian mtDNA molecules are small enough to be easily manipulated and analyzed by a variety of techniques.

Unique to mammalian mtDNA and a few viral DNA genomes is a bias in the content of guanosine plus thymine in one strand of the double helix compared to the other strand. A consequence of this situation is that the two strands of mammalian mtDNA can be physically separated to an extent that allows their individual identification. Therefore, one can isotopically label mtDNA and directly assay the extent of mtDNA synthesis that has occurred in each strand.

Perhaps the most signature feature of mammalian mtDNA is the displacement-loop (D loop). The D-loop nomenclature follows from the fact that closed-circular

mtDNA contains a region of varying size, depending on the species, in which leading-strand DNA synthesis has initiated and elongated to a size between 0.5–1 Kb. Molecules exhibiting progressive growth of this nascent leading strand, approaching full genomic size, were identified and argued as support for the proposed model for replication.

Lagging-strand synthesis is revealed by the appearance of duplex DNA within the context of the ever-increasing displaced parental-single strand. A preferred initiation site is located about two-thirds of the genomic distance away from the D-loop origin of leading-strand synthesis. Recent studies have suggested that other sites can serve as points of lagging-strand DNA replication initiation and thereby provide a broader range of duplex replicative intermediates.

## Future Studies

In contrast to studies on mtDNA, the mitochondrial system has been challenging with respect to identifying, purifying, and characterizing the critical proteins and other factors that sponsor mtDNA replication. A principal reason for this is that although low-abundance mammalian mtDNA can be successfully purified to a high state because of its closed circular physical form, in general, the mitochondrial proteins involved in mitochondrial nucleic acid syntheses tend to be similar to their much more abundant nuclear counterparts. With the current availability of mammalian genomic sequences, and cloning and expression technologies, it should be possible to make more advances in this area. A partial listing of known and predicted activities include mtDNA and mtRNA polymerases, transcription factors, single-strand DNA binding protein, specialized RNase activities, helicases, and topoisomerases.

It is now clear that mitochondrial function and the integrity of normal mtDNA are important for normal physiology in mammals. There are numerous reports of human disease in particular that have mtDNA mutations, and even loss of mtDNA, as the underlying basis for what is usually manifested as neuromuscular disorder phenotypes.With this understanding, it will be important to learn the nature and rate of mtDNA turnover in different cells and tissues. Unlike nuclear DNA, which is not replicating in nondividing cells, mtDNA continues in its need to replicate due to continuous turnover and renewal of the mitochondrial organellar population. Thus, at least the opportunity to accumulate mutations in mtDNA during an individual's lifetime can be appreciated. Knowledge of the global pattern of mtDNA-turnover rates could prove valuable in predicting risk factors for human mitochondrial disease.

There are now a number of studies that are identifying the key activities that regulate the fission and fusion of individual organelles. This will likely involve further consideration of how the mitochondrial network travels along microfilaments and is positioned within the cellular cytoplasm. In turn, it will be important to learn more about the precise location of mtDNA-replication sites to determine if replication is site-specific or can occur generally.

## Yeast and Others

Historically, the yeast system has been the most widely used to study almost all aspects of mitochondrial biogenesis. It has provided a wealth of information on bioenergetics, organelle assembly, protein import, and other areas of mitochondrial biology, including the details of mtDNA gene expression.

However, studies of mtDNA replication in yeast have been challenging due, in part, to the plasticity of the genome and the very aggressive rate of recombination of yeast mtDNA in cells. Thus the isolation and analysis of productive replicative intermediates have proved much more difficult than for mammalian systems with their small genomes and almost complete absence of recombination, including the nucleases usually associated with breakage and rejoining of DNA strands. Nevertheless, there are a few intriguing similarities between yeast and mammalian mtDNAs with regard to potential yeast mtDNA origins resembling origin sequences in D loops. It will be interesting to learn whether origins of replication have been conserved over this period of evolution, or whether other roles for these sequences, such as attachment to membranes to facilitate segregation of mtDNA molecules after replication, are the driving force for these similarities. In this regard, recent studies have suggested that mtDNA can be isolated in close association with specific proteins, which may be key to understanding genomic placement in the organelle.

## See Also the Following Articles

Mitochondrial DNA • Mitochondrial Genes and Their Expression: Yeast • Mitochondrial Genome Evolution • Nuclear Genes in Mitochondrial Function and Biogenesis

## Glossary

**closed-circular DNA** DNA in which the double helix forms a continuous circular structure with no ends nor any interruptions in either strand.

**DNA replication** The process by which a given DNA molecule duplicates itself. This process occurs in different ways depending on the form of DNA and the enzymes and factors supporting replication.

**mitochondria** The organelles in cells primarily responsible for energy production.

**mtDNA** The genome of mitochondria; every species has a characteristic mtDNA sequence.

**origin** A DNA sequence region in which new DNA synthesis begins.

**promoter** A DNA sequence recognized by enzymes and factors that copy genes into RNA molecules. Like an origin, a promoter marks the beginning of a nucleic acid synthesis event.

**transcription** The overall process by which DNA sequence is copied into RNA sequence. This is the first major step in gene expression.

## Further Reading

Attardi, G. (1986). The elucidation of the human mitochondrial genome: A historical perspective. *Bioessays* **5**(1), 34–39.

Clayton, D. A. (1982). Replication of animal mitochondrial DNA. *Cell* **28**, 693–705.

Larsson, N.-G., and Clayton, D. A. (1995). Molecular genetic aspects of human mitochondrial disorders. *Annu. Rev. Gen.* **29**, 151–178.

Scheffler, I. E. (1999). *Mitochondria*. Wiley-Liss, New York.

Shadel, G. S., and Clayton, D. A. (1997). Mitochondrial DNA maintenance in vertebrates. *Annu. Rev. Biochem.* **66**, 409–435.

## Biography

David A. Clayton is Vice President and Chief Scientific Officer of the Howard Hughes Medical Institute. Responsible for the institute's Science Department, he led the early phase of planning for the Janelia Farm Research Campus. He received his Ph.D. from the California Institute of Technology, and is a biochemist and geneticist whose research focuses on understanding the nature and role of mitochondrial DNA in cells. He is also a Professor at Stanford University and a member of the Institute of Medicine of the National Academy of Sciences.

# DNA Replication: Eukaryotic Origins and the Origin Recognition Complex

**Melvin L. DePamphilis**
*National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA*

**Cong-jun Li**
*Animal and Natural Resources Institute, US Department of Agriculture, Beltsville, Maryland, USA*

**Eukaryotic DNA replication is a highly conserved process that begins with the assembly of an origin recognition complex (ORC) composed of six different subunits (Orc1 to Orc6) at DNA replication origins distributed throughout the genome. Thus, it is the interaction between ORC and DNA that determines where DNA replication begins in the genomes of eukaryotic cells. Moreover, regulation of ORC activity is the premier step in determining when replication will occur. Pre-replication complexes (pre-RCs) are assembled at ORC/chromatin sites during the G1 phase of the cell division cycle. Pre-RCs consist of six ORC proteins, Cdc6, Cdt1, and six Mcm proteins. The role of Cdc6 is to identify ORC/chromatin sites; the role of Cdt1 is to load Mcm(2–7) hexamers onto these sites. The role of Mcm(2–7) hexamers is to unwind the DNA into two single-stranded DNA templates. DNA synthesis (S phase) is triggered by the addition of Mcm10 followed by the action of three protein kinases: Cdc7/Dbf4, Cdk2/cyclin E, and Cdk2/cyclin A. These events allow Cdc45 to escort DNA polymerase-$\alpha$:DNA primase to the pre-RC and initiate RNA-primed DNA synthesis at or close to the ORC-binding site.**

## DNA Replication Origins

### The Generic DNA Replication Origin

All DNA replication origins require two core components: (1) one or more binding sites for an origin recognition protein or protein complex, and (2) an easily unwound sequence called the DNA-unwinding element (DUE). A DUE is not sequence specific, but consists of a nucleotide composition with a low melting temperature. DNA unwinding begins within the DUE, and then DNA synthesis begins on each of the resulting single-stranded DNA templates. Additional "auxiliary" components may include one or more transcription factor binding sites that facilitate either binding of origin recognition proteins or DNA unwinding, but that are not required for origin activity. Auxiliary components are commonly found in viral replication origins. In cellular genomes, the sites where DNA replication begins, particularly in multicellular organisms, are determined by epigenetic as well as genetic parameters. This allows metazoans the flexibility to change their pattern of replication origins to accommodate changes in the length of S phase and in the pattern of gene expression that can occur during animal development. Thus, evolution has retained the same basic mechanism for DNA replication throughout the eukaryotic kingdom without sacrificing the flexibility needed in gene expression and genomic changes needed to create complex, multicellular organisms.

Eukaryotic replication origins initiate DNA replication in both directions, resulting in a transition from discontinuous to continuous DNA synthesis on each template strand (origins of bidirectional replication (OBR)) (Figure 1). This transition occurs because the two complementary DNA strands are antiparallel ($5' \rightarrow 3' : 3' \leftarrow 5'$), and all DNA polymerases travel along their template in only one direction ($5' \rightarrow 3'$). Therefore, DNA synthesis can occur continuously on one template of a replication fork in the same direction as DNA unwinding (this process is termed leading strand synthesis), but it must occur discontinuously on the complementary template, in the direction opposite to DNA unwinding, through the repeated initiation of short nascent DNA fragments called Okazaki fragments (lagging strand synthesis). Okazaki fragments are initiated by the enzyme DNA polymerase-$\alpha$:DNA primase, extended by DNA polymerase-$\alpha$:PCNA, and eventually ligated to the $5'$ end of the long growing daughter strand by DNA ligase I. The two leading strand initiation events that mark the OBR are separated by only one or two nucleotides.

Eukaryotic replication origins depend on DNA sequence information, although the requirements are less stringent than viral replication origins. Cellular

 © 2004, Elsevier Inc. All Rights Reserved. 

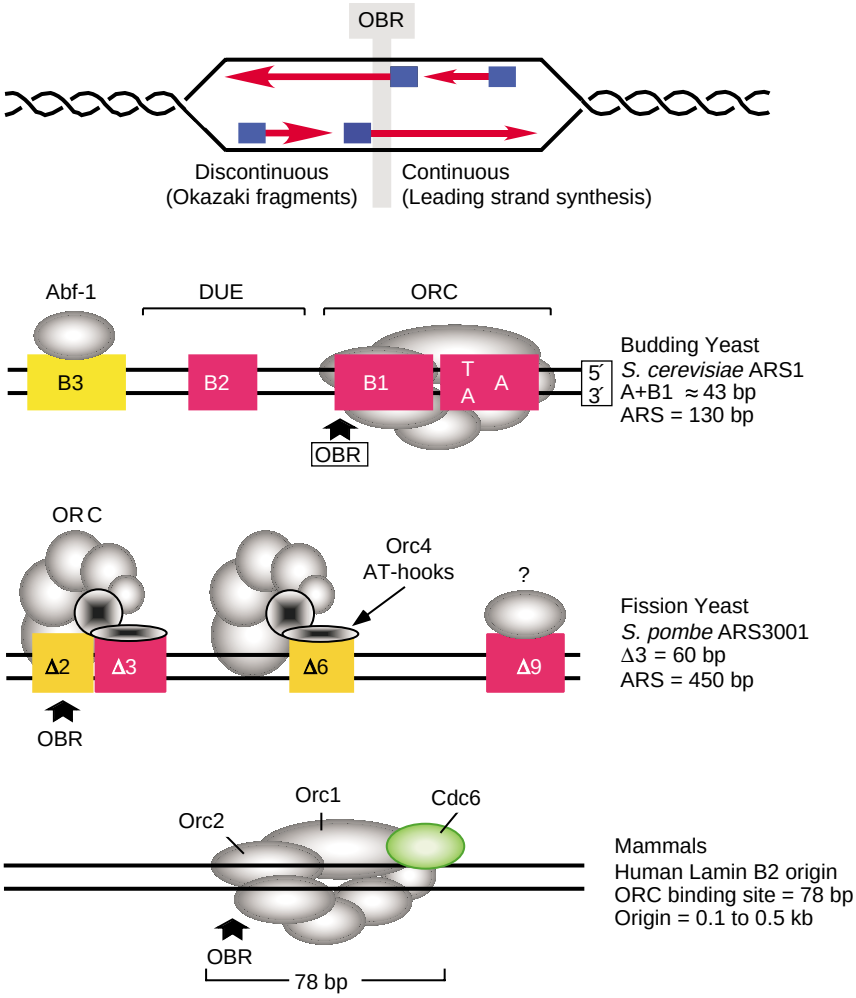



**FIGURE 1** Replication origins. Some sequence elements are required for origin activity (red) while others facilitate origin activity (yellow). The *S. cerevisiae* ARS1 origin consists of a binding site (elements A + B1) for the six subunit origin recognition complex (ORC), and a large DNA-unwinding element (DUE) that contains a genetically defined B2 element. It also contains a binding site for transcription factor Abf-1. The origin of bidirectional replication (OBR) is the site where leading strand synthesis begins on each template. The *S. pombe* ARS3001 origin consists of four genetically defined elements. Δ3 and Δ6 bind SpORC. Δ2 contains the OBR. Δ9 is required for origin activity, but its function is unknown. The human lamin B2 origin has been mapped to a ~0.6 kb site at the 3′-end of the lamin B2 gene. No genetically defined elements (replicators) have been reported, but other mammalian replicators occupy <1 kb. The OBR and DNA contact points for Orc1, Orc2, and Cdc6 have been identified.

replication origins contain a "replicator" sequence that imparts origin activity when translocated to other chromosomal sites; this can be inactivated by sequence alterations. The genomes of viruses, yeast, and protozoa also contain an autonomously replicating sequence (ARS) that allows extrachromosomal DNA (e.g., plasmids) to replicate when provided with the cognate origin recognition proteins and essential replication proteins. All ARSs exhibit replicator activity, but all replicators do not exhibit ARS activity. Such differences presumably reflect the effects of sequence context and chromatin structure on origin activity.

The most well characterized replication origins are found in the genomes of animal viruses and yeast cells. Genomes such as simian virus 40 (SV40), polyomavirus, and papillomavirus contain a single species-specific sequence of approximately 60 bp that is the binding site for the single origin recognition protein encoded by the virus. For example, SV40 encodes T-antigen, which in the presence of ATP binds specifically to the SV40 replication origin where it assembles into two hexamers with the DNA passing through their centers. The two hexamers unwind DNA in opposite directions, one hexamer for each newly formed replication fork. T-antigen hexamers assembled in the absence of DNA cannot unwind the origin. Thus, viral replication origins not only determine where replication begins, but also are required for assembly of an active helicase to initiate DNA unwinding, a prerequisite to DNA synthesis.

Yeast origins are similar to animal virus origins in that they both exhibit ARS activity. However, yeast origins differ from viral origins in four ways: (1) Viral origins initiate replication many times per cell division cycle, whereas yeast origins (like all eukaryotic origins) are activated once and only once per cell cycle. (2) Viral origins exhibit a rigid modular anatomy in which the sequence elements require a specific spacing and orientation with respect to one another, whereas yeast origins exhibit a flexible modular anatomy in which the same functional modules from different origins are interchangeable, even though they are not similar in sequence. (3) Viral origins function independently of their DNA context, whereas both the activity and the timing of the activation of yeast origins are strongly influenced by neighboring sequences. (4) Finally, viral origins are sequence-specific binding sites for a unique virally encoded origin recognition protein, whereas yeast origins exhibit little sequence specificity and bind a complex of six different proteins (ORC). ORC, in turn, recruits a helicase [Mcm(2–7)] to the replication origin.

## The Budding Yeast Paradigm

Replication begins at specific DNA sites in *Saccharomyces cerevisiae* consisting of 100 to 150 bp. The core component consists of an ScORC binding site (~30 bp) that includes two genetically identifiable elements, A and B1, as well as a DUE that usually contains the genetically identifiable B2 element (Figure 1). (Note that species is indicated by prefixes such as Sc, Sp, Xl, Dm, and Hs for *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Xenopus laevis*, *Drosophila melanogaster*, and *Homo sapiens*, respectively.) Element A contains an asymmetric A:T-rich ARS consensus sequence that is required for origin activity. B1 facilitates A in binding ScORC. B2 appears to be a weak ScORC binding site that facilitates pre-RC assembly. Some origins also contain an auxiliary component, element B3 (~22 bp), that binds transcription factor Abf-1. The OBR is marked by two start sites for leading strand DNA synthesis at specific nucleotides separated by only 1 bp. The OBR resides between the ScORC binding site and the DUE. Despite the fact that only ~15% of the sequences in *S. cerevisiae* origins are shared (parts of elements A and B1), they exhibit a flexible modular anatomy in which homologous elements from different origins are interchangeable. Yeast origins vary considerably in the frequency at which they are activated during cell proliferation and in the temporal order they are activated during S phase. Sensitivity to their DNA (chromatin?) context most likely accounts for the facts that not all ARS elements function as replication origins in yeast chromosomes and that some origins can bind ORC but still cannot initiate replication.

## The Fission Yeast Paradigm

*S. pombe* replication origins (0.5 to 1 kb) are five to ten times larger than those in *S. cerevisiae*. *S. pombe* replication origins contain ARSs that function *in vivo* as replication origins, but *S. pombe* ARSs are not interchangeable with those in *S. cerevisiae*. Furthermore, they lack a genetically required consensus sequence. *S. pombe* replication origins contain two or more regions that are required for full ARS activity. These regions consist of asymmetric A:T-rich sequences with A residues clustered on one strand and T residues on the other. Some exhibit either orientation or distance dependence, and some bind SpORC, assemble a pre-RC, and initiate bidirectional DNA replication, while others with a similar AT content do not. Thus, despite the absence of a required consensus sequence, *S. pombe* origins still exhibit sequence specificity in their design.

For example, ARS3001 (Figure 1) consists of four genetically required sites contained within ~570 bp; Δ2 and Δ6 are weakly required while Δ3 and Δ9 are strongly required. SpORC binds strongly to the Δ3 site, weakly to the Δ6 site, and not at all to the remaining sequences. Pre-RC assembly appears to occur primarily at the Δ3 + Δ2 region, and an OBR has been mapped to the Δ2 site. Thus, the Δ3 + Δ2 region (~100 bp) is equivalent to a simple *S. cerevisiae* origin. Δ6 appears similar to the B2 element in *S. cerevisiae* origins in that it is a weak SpORC binding site that facilitates origin activity. Remarkably, the Δ9 region, which is required for origin activity to the same extent as the Δ3 region, neither binds ORC nor functions as a centromere, although it does bind an as yet unidentified protein throughout the cell cycle. Therefore, Δ9 may be a novel origin component. This and other examples suggest that *S. pombe* replication origins contain at least two SpORC binding sites, consisting of asymmetric A:T-rich sequences, that act synergistically to facilitate assembly of a single pre-RC at an adjacent site.

## Metazoan Replication Origins

Replication origins in multicellular animals such as flies, frogs, and mammals differ in two critical ways from those found in animal viruses and in yeast. First, metazoan replication origins exhibit replicator activity more readily than they do ARS activity, suggesting that metazoan replication origins function better in large chromosomes than in small extrachromosomal elements. Second, metazoan ORCs do not require binding to specific DNA sequences in order to initiate assembly of a pre-RC. For example, early embryos undergoing rapid cell cleavage (e.g., frogs, flies, sea urchin, fish) initiate DNA replication with no obvious requirement for any specific DNA sequences,

and DmORC or HsORC can replace XlORC in frog egg extracts with the same result.

Nevertheless, in the differentiated cells of flies, frogs, and mammals, OBRs have been mapped repeatedly to specific genomic loci. The nature of these loci, however, is not clear. Studies employing 2D gel fractionation of total genomic DNA to detect replication bubbles or to map the polarity of replication forks generally conclude that initiation events are distributed uniformly over intergenic regions as large as 55 kb (initiation zones), whereas methods that map either the relative distribution or the relative abundance of nascent DNA strands along the genome invariably conclude that initiation events originate within specific loci of ~1 kb or smaller, similar in size to fission yeast origins. Moreover, these specific loci contain replicators that can be inactivated by internal deletions, but they lack an identifiable, genetically required consensus sequence, such as the ARS consensus sequence found in budding yeast replicators. They also contain an OBR that is comparable to those found in yeast, and at least one ORC-binding site (e.g., lamin B2) (Figure 1). In addition, origin activity can be regulated from sequences such as locus control regions that are many kilobases distal to the OBR but affect the accessibility of initiation sites to replication proteins. Thus, replication origins in mammals appear more similar to those in fission yeast than to those in budding yeast.

How might such disparate data be reconciled? The answer is that metazoan genomes contain many potential initiation sites for DNA replication, but during animal development some of these sites are selectively activated whereas others are suppressed. This is evident from the simple fact that site-specific initiation is developmentally acquired. Initiation sites are uniformly distributed throughout the genome in embryos undergoing rapid cell cleavages prior to the onset of zygotic gene expression, with no apparent preference for specific sequences. After this stage, initiation events become restricted to specific sites. Therefore, epigenetic as well as genetic parameters determine where initiation will occur.

### Epigenetic Parameters

Epigenetic parameters that can affect origin activity include nucleotide pool levels, transcription factor binding sites, concentration of replication proteins, transcription, chromosome structure, nuclear organization, and DNA methylation. For example, under normal culture conditions, one particular 128 kb locus in the hamster cell genome initiates predominately at a single primary (high-frequency) origin. However, reducing dNTP pools distributes initiation events equally among six different origins within this locus (the primary origin plus five secondary or low frequency origins). This implies that synchronization of cells at their G1/S boundary by reducing nucleotide pools would favor the appearance of initiation zones, a caveat that may account for some of the data in the literature. It also implies that the frequency of initiation sites in mammals is similar to the frequency in yeast and in frog eggs (1/20 to 1/30 kb).

Similarly, transcription factors binding close to ORC-binding sites can facilitate origin activity. Because they are expressed in specific cells and at specific times during development, transcription factors could impart both developmental and DNA site specificity to origins. High ratios of ORC to DNA should favor initiation at low- as well as high-affinity DNA binding sites, whereas low ratios would favor initiation at high-affinity sites only. Since the concentration of ORC in the eggs of the frog, *Xenopus laevis*, is about $10^5$ greater than in frog somatic cells, this could account for the transition from "random" to site-specific initiation events that is observed during frog development. In addition, the onset of zygotic gene expression will repress initiation events in the transcribed regions, because RNA synthesis through replication origins represses their activity. Similarly, changes in chromosome structure will make some genomic sites more accessible to ORC while making others less accessible. Changes in nuclear organization may also contribute to origin specification, because site-specific initiation of DNA replication has been observed only with intact cells or with intact (impermeable) nuclei incubated in cell extracts. Finally, DNA methylation is associated with repressed chromatin, and changes in DNA methylation patterns have been correlated with changes in initiation site activity.

Primary and secondary origins clearly exist in yeast, where some origins are activated once each cell division cycle while others are not, and in bacteriophage such as T7, where deletion of the primary origin simply shifts replication to a secondary origin. Most mammalian origins contain AT-rich sequences of the type commonly found at matrix attachment regions and in the replication origins of fission yeast and flies. In fact, ORCs from *S. pombe*, *Xenopus* eggs, and human cells all target asymmetric A:T-rich sequences. Perhaps their affinity for a particular site depends on epigenetic factors.

## The Origin Recognition Complex

Eukaryotic ORCs are functionally, if not structurally, conserved among yeast, frogs, flies, and mammals. They all bind to DNA with nanomolar affinity, and they all bind preferentially to asymmetric A:T-rich sequences. ORC subunits 1, 4, and 5 bind ATP. The ATP-binding site in Orc1 is required for ORC

activity, and ORC exhibits ATPase activity. Only two of the six ORC subunits contain multiple consensus sites for phosphorylation by cyclin-dependent protein kinases, suggesting that these subunits are targets for regulation. These are Orc1 and Orc2 in the metazoa and Orc2 and Orc6 in yeast. Nevertheless, there are notable differences.

The ability of ORC to bind specific DNA sequences appears to be species dependent. Site-specific DNA binding by *S. cerevisiae* ORC requires subunits 1 to 5 and ATP; once ScORC is bound to origin DNA, it remains there throughout the cell division cycle. ScORC covers ~80 to 90 bp, and origin binding is facilitated by Cdc6. ScORC bound to dsDNA origins exists in an extended conformation, whereas ScORC bound to ssDNA forms a bent conformation. In addition, ScORC ATPase activity is stimulated by ssDNA but inhibited by dsDNA. Thus, it appears that *S. cerevisiae* double-stranded origin DNA, ATP, and Cdc6 stabilize bound ScORC in a conformation that allows pre-RC assembly, whereas ssDNA, ATP hydrolysis, and the loss of Cdc6 converts ScORC into a form that may release the Mcm(2–7) helicase to continue unwinding DNA at replication forks.

Like *S. cerevisiae*, all six *S. pombe* ORC subunits remain tightly bound to chromatin throughout the cell cycle. In contrast to *S. cerevisiae*, however, site-specific DNA binding by *S. pombe* ORC requires only the SpOrc4 subunit; neither the presence nor the absence of ATP and the other five subunits affects SpOrc4 binding to DNA *in vitro*. The SpOrc4 subunit is unique among eukaryotes in that its N-terminal half contains nine AT-hook motifs that specifically bind the minor groove of AT-rich DNA. The C-terminal half of SpOrc4 is 35% identical and 63% similar to the human and *Xenopus* Orc4 proteins. SpOrc4 has a general affinity for all AT-rich DNA, but it has a higher affinity for specific asymmetric A:T-rich sequences found within *S. pombe* replication origins. In fact, chromatin immunoprecipitation assays reveal that SpORC is bound to *S. pombe* origins and not to other AT-rich sequences in the regions between origins. Thus, while each AT-hook motif binds tightly to [AAA(T/A)], site specificity likely results from the arrangement of all nine motifs acting in concert.

Whereas the six ORC subunits in yeast and frogs form a stable complex *in vitro*, human ORC consists of a stable ORC[2–5] subcomplex to which Orc1 and Orc6 are only weakly bound. Both frog and human ORC binds preferentially to asymmetric A:T-rich sequences *in vitro* or during DNA replication in frog egg extracts, and the sequences selected by these metazoan ORCs are the same as those targeted by SpOrc4. Nevertheless, HsORC is localized at specific DNA replication origins *in vivo*.

## Regulating ORC Activity: The ORC Cycle

One universal feature of eukaryotic DNA replication is that the genome is replicated once and only once each time a cell divides. This is accomplished in two ways. First, pre-RCs that are assembled during the M to G1 phase transition are inactivated during S phase, and second, new pre-RCs cannot be assembled until G1 phase. This is accomplished by blocking pre-RC assembly and activation at multiple steps such as the Cdc6, Cdt1, Mcm(2–7), and Cdk2 functions. However, the premier step in determining both where and when DNA replication begins is the assembly of functional ORC/chromatin sites, and this step appears to be regulated by inactivating ORC during the G1 to S phase transition and then preventing re-establishment of ORC activity until mitosis is completed and, in the metazoa, a nuclear membrane is reassembled.

In contrast to mammals and frogs, all six ORC subunits in yeast remain tightly bound to chromatin throughout their cell division cycles. Nevertheless, yeast ORC subunits undergo cell cycle-dependent phosphorylation that contributes to preventing reinitiation of DNA replication before cell division is finished. Orc2 and Orc6 are phosphorylated by Cdk1(Cdc28)/cyclin B during the S to M transition, and then dephosphorylated during early G1 phase when pre-RC assembly occurs. In *S. pombe*, Cdk1(Cdc2)/cyclin B associates with replication origins during S phase and remains there during G2 and early M phases. This association is ORC dependent and prevents reinitiation of DNA replication before mitosis has been completed (Figure 2).

In frog eggs, all six ORC subunits remain stably bound to one another throughout the cell division cycle, but their affinity for chromatin is cell cycle dependent. When sperm chromatin replicates in a *Xenopus* egg extract, the affinity of XlORC for chromatin becomes salt-labile following pre-RC assembly and then appears to be released during G2/M phase as a result of hyperphosphorylation by Cdk1/cyclin A. However, when somatic cell chromatin replicates under the same conditions, XlORC binds to the chromatin, initiates pre-RC assembly, and is then released upon completion of pre-RC assembly. Thus, the affinity of XlORC for chromatin depends on at least three factors: pre-RC assembly, chromatin structure, and the action of Cdk1/cyclin A (Figure 2).

In mammalian cells, ORC subunits two to five remain bound to chromatin throughout the cell division cycle, but the affinity of the largest subunit, Orc1, for chromatin is selectively reduced during S phase and then restored during the M to G1 phase transition (Figure 3). During G1 phase, Orc1, Orc2, Cdc6, and Mcm3 proteins can be cross-linked to replication origins (e.g., lamin B2

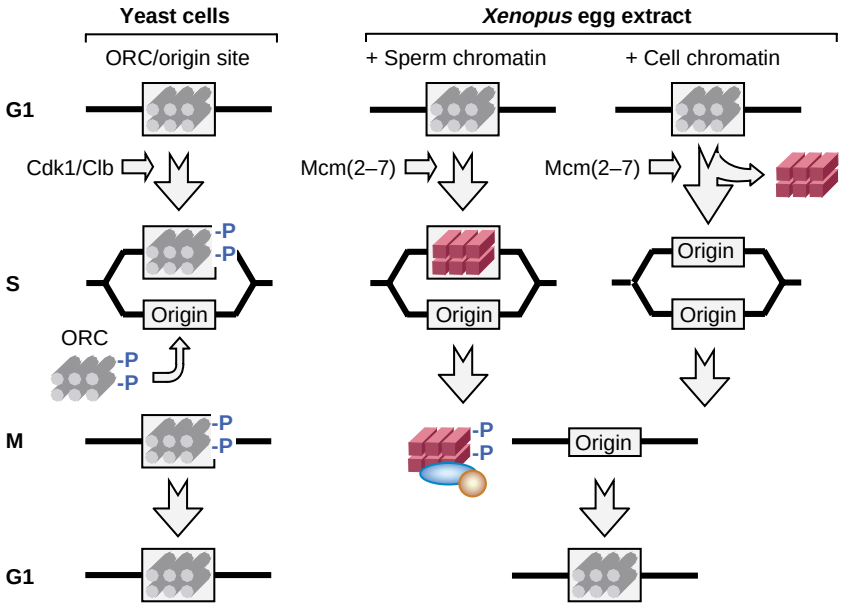



**FIGURE 2** Three manifestations of the ORC cycle in eukaryotes. Yeast cells: ORC (six gray cylinders) remains bound to replication origins throughout the cell cycle, but ORC is phosphorylated (-P) during the S to M periods; this phosphorylation inhibits its ability to assemble a pre-RC. *Xenopus* egg extract: ORC binds to sperm chromatin, but the stability of ORC/chromatin sites is reduced (red boxes) following pre-RC assembly. ORC is phosphorylated by Cdk1/cyclin A (yellow ball) during G2/M and released from chromatin. If somatic cell chromatin is incubated in the extract instead of sperm chromatin, then ORC is released from chromatin following pre-RC assembly.

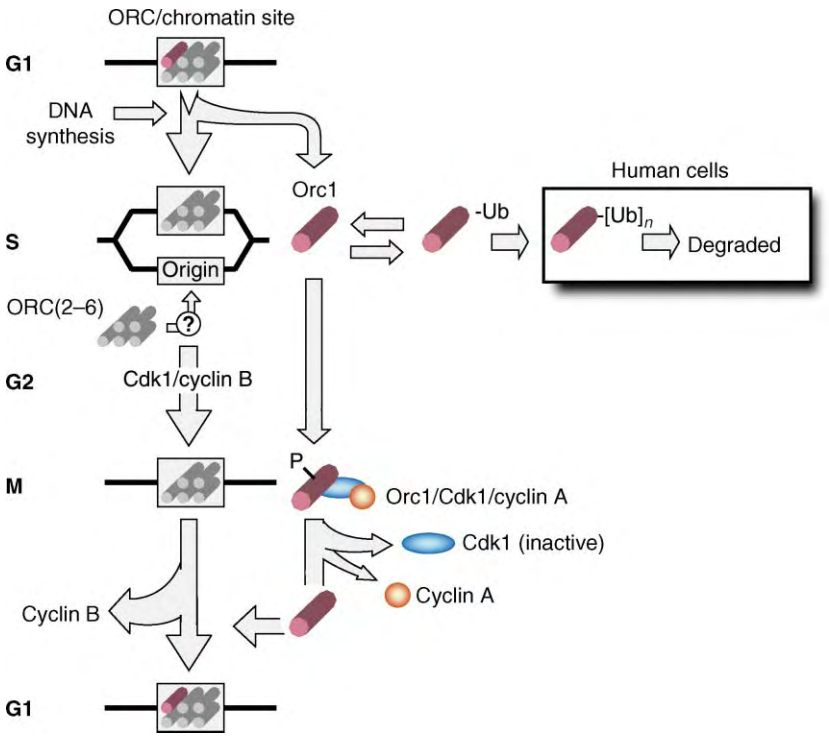



**FIGURE 3** The ORC cycle in mammalian cells. ORC subunits 2 to 6 (grey cylinders) remain bound to chromatin throughout the cell cycle, but the Orc1 subunit (red cylinder) is selectively destabilized and released from chromatin when DNA synthesis begins (S phase). Orc1 is then mono-ubiquitinated (Ub) and in some cases polyubiquitinated ($[Ub]_n$) and degraded. Orc1 in mitotic cells is hyperphosphorylated by its association with Cdk1/cyclin A. When this enzyme is inhibited during the M to G1 transition, the phosphorylated state of Orc1 is reduced and it binds to chromatin.

origin; Figure 1), but during S phase, only the Orc2 protein can be cross-linked, consistent with release of Orc1 and disassembly of a pre-RC and with the fact that mammalian metaphase chromatin lacks functional ORCs. Rebinding of Orc1 to chromatin follows the same time course as degradation of cyclin B, suggesting that exiting mitosis triggers Orc1 binding to chromatin. Furthermore, Orc1 binding to chromatin precedes the appearance of functional pre-RCs at specific origins of bidirectional replication, suggesting that assembly of functional ORC/chromatin sites is the rate-limiting step in the assembly of pre-RCs at specific genomic sites.

What prevents Orc1 from rebinding to chromatin? In human cells, most of the Orc1 is selectively released, polyubiquitinated, and degraded during S phase. However, in hamster cells, the Orc1 is released but it is mono-ubiquitinated and not degraded *in vivo*. Moreover, during the S to M transition in hamster cells, mono-ubiquitinated Orc1 is replaced by Orc1, and the Orc1 in G2/M phase cells is hyperphosphorylated by its association with Cdk1/cyclin A (Figure 3). Inhibition of protein kinase activity in M phase cells allows dephosphorylation of Orc1 and rapid reassociation of Orc1 with chromatin. The association of Orc1 with chromatin appears to be the rate-limiting step in assembly of functional pre-RCs during the M to G1 phase transition. Thus, there is a universal control point in eukaryotic cell division cycles: the same cyclin-dependent protein kinase that regulates the onset of mitosis (Cdk1) also prevents premature assembly of functional ORC/chromatin sites until mitosis is complete and a nuclear membrane is present.

## See Also the Following Articles

Cell Cycle: Control of Entry and Progression Through S Phase • Chromatin: Physical Organization • Chromatin Remodeling • DNA Replication Fork, Eukaryotic • Nuclear Organization, Chromatin Structure, and Gene Silencing

## Glossary

**autonomously replicating sequence (ARS)** A DNA sequence that imparts origin activity to extrachromosomal elements in the presence of appropriate replication proteins, and whose activity is sensitive to genetic alterations.

**Cdc** Cell division cycle protein. A large number of Cdc genes have been identified in eukaryotes that affect various stages in cell division. Cdc6 is the name used in the budding yeast, *Saccharomyces cerevisiae*; Cdc18 is the name used in the fission yeast, *Schizosaccharomyces pombe*. The nomenclature for budding yeast proteins is generally applied to other organisms.

**Cdk** Cyclin-dependent protein kinase. This phosphorylates specific amino acids in proteins, but only when associated with a cyclin protein.

**Cdt1** A protein encoded by Cdc10-dependent transcript 1 in *S. pombe*. Cdt1 is the same as RLF-B in *Xenopus laevis*.

**DNA replication origin** The DNA site where replication begins; also called an origin of bidirectional replication.

**Mcm** Minichromosome maintenance proteins. These were identified as genes required to maintain plasmids in *S. cerevisiae*. At least seven of these proteins are involved in DNA replication.

**origin recognition complex (ORC)** Six different proteins that bind to DNA replication origins and thereby initiate assembly of a pre-replication complex.

**pre-replication complex (pre-RC)** Fourteen different proteins consisting of ORC, Cdc6, Cdt1, and Mcm(2–7) that form a complex with chromatin during G1 phase of the cell cycle and that become the site where DNA replication begins during S phase.

**replicator** A DNA sequence that imparts origin activity when translocated to other chromosomal regions, and whose activity is sensitive to genetic alterations.

## Further Reading


Abdurashidova, G., Danailov, M. B., Ochem, A., Triolo, G., Djeliova, V., Radulescu, S., Vindigni, A., Riva, S., and Falaschi, A. (2003). Localization of proteins bound to a replication origin of human DNA along the cell cycle. *EMBO J.* **22**, 4294–4303.

Anglana, M., Apiou, F., Bensimon, A., and Debatisse, M. (2003). Dynamics of DNA replication in Mammalian somatic cells. Nucleotide pool modulates origin choice and inter-origin spacing. *Cell* **114**, 385–394.

Bell, S. P. (2002). The origin recognition complex: From simple origins to complex functions. *Genes Dev.* **16**, 659–672.

Bell, S. P., and Dutta, A. (2002). DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71**, 333–374.

Bogan, J. A., Natale, D. A., and DePamphilis, M. L. (2000). Initiation of eukaryotic DNA replication: Conservative or liberal? *J. Cell. Physiol.* **184**, 139–150.

DePamphilis, M. L. (1999). Replication origins in metazoan chromosomes: Fact or fiction? *Bioessays* **21**, 5–16.

DePamphilis, M. L. (2003). The 'ORC cycle': A novel pathway for regulating eukaryotic DNA replication. *Gene* **310**, 1–15.

Kong, D., Coleman, T. R., and DePamphilis, M. L. (2003). *Xenopus* origin recognition complex (ORC) initiates DNA replication preferentially at sequences targeted by *Schizosaccharomyces pombe* ORC. *EMBO J.* **22**, 3441–3450.

Li, C-J., Vassilev, A., and DePamphilis, M. L. (2004). A role for Cdk1(Cdc2)/Cyclin A in preventing the mammalian Origin Recognition Complex's largest subunit (Orc1) from binding to chromatin during mitosis. *Mol. Cell. Biol.*, in press.

Prioleau, M. N., Gendron, M. C., and Hyrien, O. (2003). Replication of the chicken beta-globin locus: Early-firing origins at the 5′ HS4 insulator and the rho- and betaA-globin genes show opposite epigenetic modifications. *Mol. Cell. Biol.* **23**, 3536–3549.

Takahashi, T., Ohara, E., Nishitani, H., and Masukata, H. (2003). Multiple ORC-binding sites are required for efficient MCM loading and origin firing in fission yeast. *EMBO J.* **22**, 964–974.

Vashee, S., Cvetic, C., Lu, W., Simancek, P., Kelly, T. J., and Walter, J. C. (2003). Sequence-independent DNA binding and replication initiation by the human origin recognition complex. *Genes Dev.* **17**, 1894–1908.


## Biography

Melvin L. DePamphilis began his career in the field of DNA replication as a postdoctoral fellow with Paul Berg at Stanford University Medical School, and continued in this field as a Professor at Harvard Medical School, an Adjunct Professor at Columbia Medical School, a member of the Roche Institute of Molecular Biology, and most recently a Section Chief at the National Institutes of Health. He has published over 150 papers, reviews, and books on the subject of DNA

replication, chromatin structure, and gene expression in a variety of experimental systems.

Cong-jun Li began his career in the field of DNA replication as a graduate student with the late Earl F. Baril at the Worcester Foundation for Experimental Biology and continued in this field as a postdoctoral fellow with Paul T. Englund at the Johns Hopkins School of Medicine and then with Melvin L. DePamphilis at the National Institutes of Health. He has published over 20 papers and reviews on DNA replication and related subjects. Currently he is a Principal Investigator at the Animal and Natural Resources Institute, a division of the U.S. Department of Agriculture.

# DNA Replication: Initiation in Bacteria

**Jon M. Kaguni**
*Michigan State University, East Lansing, Michigan, USA*

**The study of *Escherichia coli* as a model organism has led to significant advancements in understanding the molecular mechanisms of DNA replication. This bacterium carries a circular duplex genome of $4.7 \times 10^6$ bp, and DNA replication initiates from a single replication origin, *oriC*. This locus is the site at which the duplex DNA opens to become single stranded. The replication fork machinery then assembles at this site, and moves bidirectionally as it copies the parental DNA to terminate chromosomal DNA replication in a region 180° opposite *oriC*. The daughter chromosomes are then segregated to progeny cells at cell division.**

## The *E. coli* Replication Origin, *oriC*

*Escherichia coli oriC* was identified genetically, then more precisely mapped by molecular DNA methods. Its isolation was based on its ability to confer autonomous replication to a plasmid formed by joining *oriC* to a DNA fragment that conferred drug resistance and lacked a replication origin. Subsequently, *oriC* was studied extensively by mutational analysis which, together with biochemical studies, showed that it carries five similar 9 bp motifs called DnaA boxes that are bound by DnaA protein, the initiator of bacterial chromosomal replication (see Figure 1). Each of the DnaA boxes (R1–R5) and their orientation relative to one another is critical for the function of *oriC* as the replication origin. For example, insertions are not tolerated in the region between the AT-rich region and R1, R5, and R1 or R5 and R2, indicating that the spacing between respective sequence elements in the left half of *oriC* must be rigorously preserved. In the right half, the spacing can be varied as long as the helical phasing of DnaA boxes is maintained. Collectively, these results suggest that the geometry of DnaA protein monomers relative to the DnaA boxes is critical for the initiation process.

*oriC* also contains binding sites for factor for inversion stimulation (FIS) and integration host factor (IHF). Mutation of these sites inactivates *oriC*, confirming their functional importance. IHF acts directly in initiation by stimulating DnaA-dependent opening of an AT-rich region of *oriC*, perhaps by bending the DNA to bring DnaA bound to DnaA boxes near the AT-rich region to support opening (Figure 1). Alternatively, IHF may act by binding to the unwound single-stranded DNA to stabilize it. IHF also contributes to the proper timing of initiation. Whereas wild type strains growing exponentially initiate new rounds of DNA replication synchronously, a null mutant of *himA*, encoding one of the subunits of the IHF heterodimer, is defective in this timing.

Similarly, *fis* mutants are asynchronous in initiation, and maintain *oriC* plasmids poorly at temperatures $\geq 37$°C. Furthermore, deletion of DnaA box R4 at the chromosomal *oriC* locus, but not when *oriC* is carried in a plasmid, can only be tolerated when the *fis* gene is functional. Whereas these observations suggest a positive role for FIS, it is not required *in vitro* for *oriC* plasmid replication. Instead, FIS is inhibitory by sequestering the negative superhelicity that is essential for *oriC* plasmid replication.

IciA protein binds to 13-mer sequence motifs in the AT-rich region of *oriC* to prevent DnaA-dependent unwinding of this region. Because *iciA* null mutants are viable and do not show an asynchrony phenotype, the physiological importance of IciA as a regulator of initiation is uncertain.

Finally, *oriC* has eleven copies of the sequence GATC which is recognized by DNA adenine methyltransferase (Dam). This enzyme acts in mismatch repair in the discrimination of the parental DNA from the unmethylated progeny DNA strand after a cycle of DNA replication. The GATC sites in *oriC* remain hemimethylated for about one-third of the cell cycle whereas most other sites are methylated much more rapidly. Hemimethylated *oriC* associates with proteins in the inner membrane, including SeqA and SeqB to prevent premature reinitiation.

 © 2004, Elsevier Inc. All Rights Reserved. 

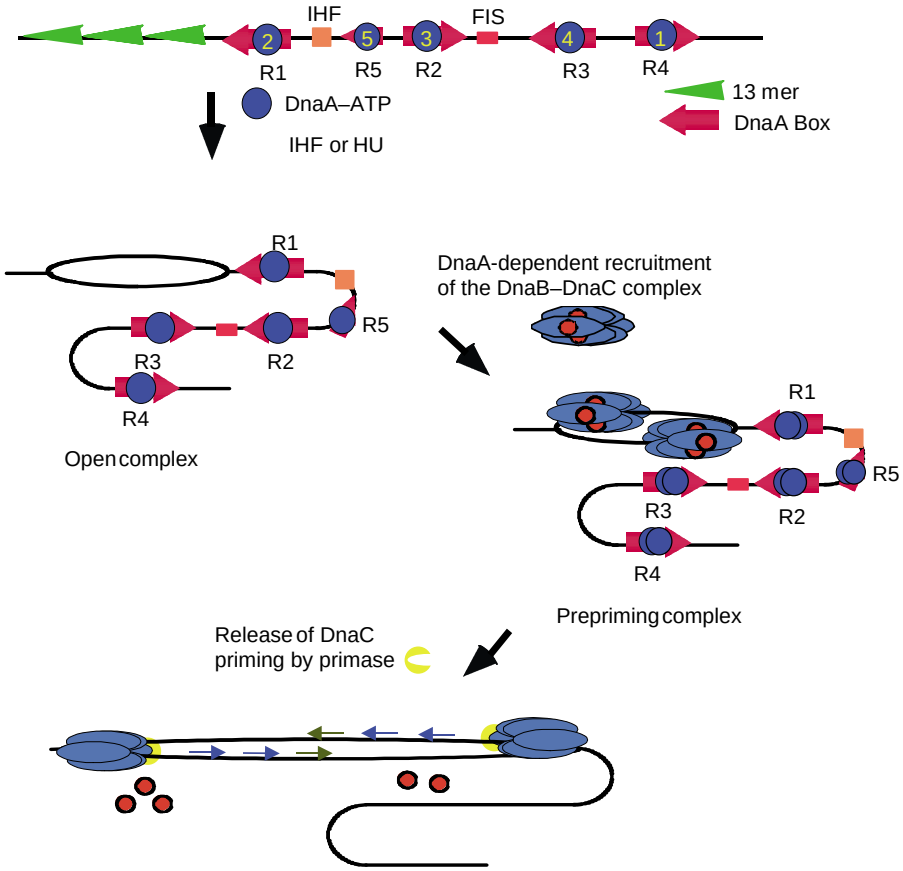



**FIGURE 1** Mechanism of initiation of DNA replication from the *E. coli* replication origin, *oriC*. Sequence elements in *oriC* include the DnaA boxes (red arrows), 13-mer motifs (green triangles) in the AT-rich region near the left border, and binding sites for IHF and FIS. In the first step, DnaA protein binds to respective DnaA boxes sequentially, as indicated by the superimposed numbers. Complexed to ATP, DnaA then opens an AT-rich region that contains 13-mer sequences near the left *oriC* boundary to form the open complex. Entry of hexameric DnaB (blue ellipse) from the DnaB–DnaC complex follows to assemble the prepriming complex. In the import of DnaB to *oriC*, DnaB initially binds to *oriC*-bound DnaA protein. The hydrolysis of ATP bound by DnaC (red circle) in the DnaB–DnaC complex releases DnaC. Bound to each parental DNA template, DnaB then moves in the 5′–3′direction and interacts periodically with primase, which synthesizes RNA primers necessary for both leading and lagging strand synthesis. These primers are then extended by DNA polymerase III holoenzyme during semiconservative DNA replication.

## Mechanism of Initiation at *oriC*

The molecular mechanism describing initiation of DNA replication from *oriC* is based primarily on the study of plasmids carrying this sequence. Such plasmids are useful model systems because its DNA replication resembles that which occurs at the chromosomal *oriC* locus. First, *oriC* plasmids replicate in synchrony with the bacterial chromosome. Second, they are duplicated with bidirectional fork movement. Third, *oriC* plasmids require the same gene products needed for copying the bacterial genome. Purified enzyme systems have been developed that sustain DNA replication on a plasmid carrying the *oriC* sequence. This has led to the biochemical analysis of the replication process, including the function of DnaA protein, the replication initiator.

### DnaA Protein and the DnaA Box

DnaA protein performs a number of essential functions in initiation, including recognition of each DnaA box sequence in *oriC*. DNA binding assays first showed that DnaA specifically recognizes *oriC* as well as to other DNAs carrying the DnaA box sequence. DNase I foot-printing revealed that DnaA protected the DnaA box in addition to flanking sequences. In *oriC*, DnaA binds to the five boxes with different affinities, apparently as a monomer to each site. Whereas DnaA box R3 is the weakest site, DnaA must be bound to it and to the other DnaA boxes to promote initiation *in vitro*. This finding corroborates *in vivo* foot-printing studies showing that DnaA bound to R1, R2, and R4 throughout the cell cycle, but that it bound to DnaA box R3 only at the time of initiation.

### Unwinding of the AT-Rich Region

DnaA is a nucleotide binding protein with a preference for ATP. In a complex with ATP and bound to *oriC*, DnaA induces unwinding of an AT-rich region near the left border of *oriC*. Whereas DnaA alone can induce this topological alteration, IHF stimulates this reaction, perhaps by binding to the IHF site in *oriC*. HU, a small basic protein that binds with higher affinity to nicked, gapped or cruciform DNA than to duplex DNA or RNA can replace IHF, and may stabilize the region of single-stranded DNA opened by DnaA. If so, IHF may similarly act to stabilize the single-stranded DNA as it is similar to HU in amino acid sequence.

DnaA is a weak ATPase, but ATP hydrolysis is not required for unwinding because a nonhydrolyzable ATP analogue is as effective as ATP in supporting strand opening of *oriC* and initiation. As DnaA bound to ADP is far less active in both unwinding and initiation, a nucleoside triphosphate or its analogue is required. A discrepancy is that the binding affinity ($K_d$) for ATP of 0.03 μM is much greater than the 1–5 mM ATP that is optimal for the unwinding reaction. It is likely that the complex of DnaA bound to *oriC* is reduced in its affinity for ATP.

### Helicase Recruitment

Following the unwinding of *oriC*, DnaA then directs the binding of two DnaB helicase molecules to *oriC* to assemble the prepriming complex. The function of DnaB as a helicase is to unwind the parental duplex DNA so that it can be copied by the replication fork machinery. Helicase recruitment involves a physical interaction between DnaA and DnaB in the DnaB–DnaC complex. The interaction of DnaA with DnaB is interesting to consider in the context of the orientation of DnaA boxes in *oriC* and in plasmid replicons that require DnaA, DnaB, and DnaC protein for DNA replication. In these plasmids that replicate unidirectionally, the DnaA boxes can be viewed to "point" in the direction of replication fork movement. In *oriC*, four of the boxes are arranged as two sets of repeats that point in both directions, corresponding to the bidirectional mode of replication fork movement. An attractive model is that DnaA orients the binding of DnaB at *oriC* so that it is pointed in the proper direction for replication fork movement.

If DnaA orients the binding of DnaB to *oriC*, inverting the DnaA boxes should affect the directionality of replication fork movement. However, inverting DnaA boxes R1, R2, and R4 individually abolished *oriC* function, probably because this perturbs the architecture of the nucleoprotein complex. Recall that the spatial arrangement of the DnaA boxes relative to each other is critical for *oriC* function.

Hexameric DnaB of identical subunits is a ring-shaped molecule with a central cavity. One of the two strands of duplex DNA passes through the central cavity of DnaB as it unwinds DNA. For its assembly into the prepriming complex, DnaC transiently bound to the unwound region of *oriC* via its cryptic single-stranded DNA-binding activity may assist at this step in loading DnaB onto the DNA. ATP hydrolysis is then required to liberate DnaC from DnaB, a necessary step for DnaB to act as a helicase else the helicase activity of DnaB is suppressed.

### Events after Prepriming Complex Formation

Upon the delivery of DnaB to *oriC*, DnaB is first bound to the unwound region of *oriC*. DnaB then unwinds the parental duplex, driven by ATP hydrolysis. Primase transiently interacts with the translocating DnaB to synthesize primers that are extended by DNA polymerase III holoenzyme. Single-strand DNA-binding protein binds to the single-stranded DNA formed by DnaB helicase activity. DNA gyrase and topoisomerase IV relieve the positive superhelicity created ahead of the replication fork. To process the Okazaki fragments formed on the lagging strand template, DNA polymerase I removes the RNA primers by nick translation, and DNA ligase seals the resulting discontinuities to make the progeny DNA fully duplex.

## Functional Domains of DnaA Protein

The alignment of many *dnaA* homologues led to the proposal that DnaA is composed of four structural domains (see Figure 2). A small region of moderate sequence conservation near the N terminus is followed by a region of variable length that is species-dependent and not conserved. The C-terminal two-thirds of DnaA protein is highly conserved in this broad range of species. Most notable is the absolute conservation of the P-loop motif or Walker A box (residues 172–179 in *E. coli* DnaA) involved in nucleotide binding. The region of DnaA carrying this portion forms a Rossmann fold by the EMBL PHD method of secondary structure prediction.

In addition to these in silico observations, characterization of a large collection of *dnaA* alleles led to the identification of functionally distinct domains that correspond well with the domain structure derived from sequence homology, and from the crystal structure of the C-terminal two-thirds of *Aquifex aeolicus* DnaA. As a brief summary, domain I functions in self-oligomerization and in retention of DnaB in the

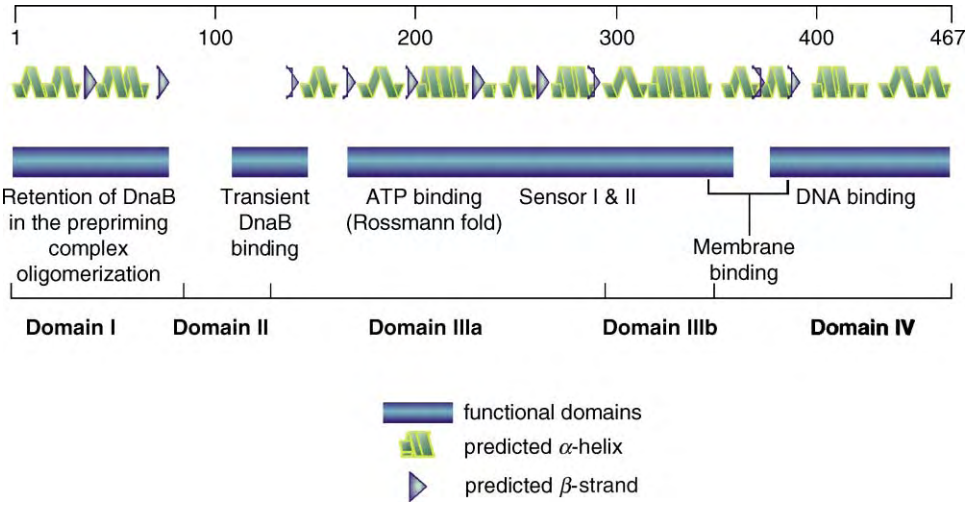



**FIGURE 2** Functional domains of DnaA protein. The top line represents the primary sequence of DnaA protein of 467 amino acids. The predicted secondary structure is below, with $\alpha$-helices in yellow and $\beta$-strands in purple. DnaA is a member of the $AAA^+$ family of proteins (ATPases associated with a variety of cellular activities) which have Walker A and B motifs involved in nucleotide binding and hydrolysis, and the sensor 1 and sensor 2 motifs that may function to sense nucleotide binding and hydrolysis. The Walker A motif is also known as the P-loop. The colored diagram near the bottom represents functional domains of DnaA in comparison to the domain structure of DnaA predicted by amino acid sequence alignment, and confirmed by analysis of the crystal structure of *A. aeolicus* DnaA protein.

prepriming complex. Domain II varies in length and sequence among bacteria and apparently functions as a flexible linker because an in-frame deletion of amino acids 87–104 of *E. coli* DnaA does not affect replication activity. A region overlapping domain II and III interacts with DnaB, and is involved in loading of the helicase at *oriC*. Domain III contains the Walker A and B boxes, and the sensor I and II motifs shared among $AAA^+$ proteins that typically bind ATP to regulate protein function. ATP binding induces a conformational change of DnaA, and mutant proteins bearing amino acid substitutions in or adjacent to the Walker A box are defective in ATP-binding activity. Domain IV carries a helix-turn-helix motif that recognizes nucleotides in the major groove of the DnaA box sequence via amino acids in the DnaA signature sequence. Near the N-terminal border of domain IV, a region involved in phospholipid binding has been described.

## Regulation of DNA Replication in *E. coli*

DNA replication is regulated at the step of initiation at *oriC*. For example, *E. coli* cultures can have generation times that vary over a tenfold range from 20 to 200 min. In cultures growing faster than 60 min, the time needed to duplicate the genome is relatively constant at about 40 min. For a culture with a 20 min generation time, new initiations occur before the replicating chromosome is completed. In cells growing more slowly, the time needed to duplicate the chromosome is interspersed by a period when no DNA replication occurs. Under these conditions, replication forks move at constant speed due to the concerted actions of DnaB helicase and DNA polymerase III holoenzyme. Thus, DNA replication is controlled by modulating the frequency of initiation, occurring more often in rapidly growing cells compared to more slowly growing *E. coli*.

Because DNA replication in *E. coli* and all other free-living organisms is a periodic event in the cell cycle, factors act to regulate DNA replication either prior to or after initiation to prevent DNA synthesis from occurring at the wrong time. Several concepts have emerged from physiological studies in bacteria. One is initiation mass, the ratio of cell mass per replication origin. This ratio is a constant value, and DnaA protein contributes to setting this ratio. Other cellular factors that govern how this ratio is kept constant have not been identified. Thus our understanding of this process is rudimentary. A second concept is initiation synchrony. In rapidly growing cultures, individual cells initiate additional rounds of DNA replication synchronously, as measured by flow cytometry under conditions in which cell division and new initiations are blocked, but ongoing DNA replication proceeds to completion, so that cells carry $2^n$ chromosomes where $n \geq 0$ and is an integer. In contrast, asynchronous DNA replication results in odd numbers of chromosomes ($1^n$) as observed in mutants carrying *dnaA*(Ts) alleles defective in ATP binding, suggesting that ATP binding by DnaA

is necessary for proper regulation of initiation. Other mutations in *fis*, *himA*, *seqA*, *dam*, *hupA*, and *hda* also result in an asynchrony phenotype because the respective mutations perturb the initiation process by direct or indirect effects.

## DnaA Protein and the Regulation of Chromosomal Replication

Among the genes required for *E. coli* DNA replication, the *dnaA* locus is unique in that conditionally defective mutations specifically affect initiation of DNA replication. By comparison, mutations in the *dnaB* and *dnaC* genes result in either an elongation- or initiation-defective phenotype, consistent with biochemical studies which show their dual roles in initiation at *oriC* and during elongation. All other replication genes display an elongation-defective phenotype because the respective proteins function in progression of the replication fork.

Several observations indicate that DnaA protein acts as a regulatory factor in the initiation process. First, DnaA protein appears to be limiting for initiation because its overproduction increases the frequency of initiation. In one study, the *dnaA*$^+$ gene (plasmid borne) was expressed from a regulated promoter in a *dnaA*(Ts) strain at nonpermissive temperature, causing initiation to be dependent on the level of induced expression. At an intermediate level of expression (13%), the synchrony of replication was comparable to the wild type control. Apparently, cyclic variation of DnaA protein is not required for initiation synchrony despite experiments showing that expression of the *dnaA* gene is autoregulated, to maintain a relatively constant cellular level of DnaA. Recent evidence implicates the *datA* locus in regulating initiation by controlling the availability of DnaA. A second line of evidence is the hyperactive initiation promoted by a mutant *dnaA* allele named *dnaAcos*. Recent experiments show that the replication activity of DnaA$^+$ protein is normally regulated to prevent unscheduled initiations whereas DnaAcos fails to respond to negative regulation. Third, some *dnaA* mutations at permissive temperature display an increased initiation mass. As the mutant proteins have reduced initiation activity *in vitro*, the lower frequency of initiation *in vivo* may lead to an increased initiation mass by promoting initiation later in the cell cycle. Together, these results suggest that DnaA protein activity controls initiation in the cell cycle.

## The *datA* Locus

Interestingly, a 1 kbp chromosomal segment called *datA* is one of eight sites in the *E. coli* genome that are bound by DnaA with high affinity. The *datA* locus carries five DnaA boxes to which several hundred DnaA monomers can bind. Strains lacking this chromosomal segment exhibit both asynchronous initiations as well as an increased frequency of initiation. The *datA* site is unique among the eight strong binding sites for DnaA in that its deletion and not others leads to aberrant initiation. Apparently, when the level of DnaA is elevated that would otherwise lead to increased initiations, the extra DnaA can bind to the *datA* locus, thus modulating the initiation process.

## SeqA at *oriC*

*E. coli* DNA replication occurs only once per cell cycle. Several proteins have been identified that bind to newly duplicated origins to block premature initiations. Specific binding to the newly replicated origins is by virtue of hemimethylated GATC sequences, as exists when the progeny DNA strand annealed to the methylated parental DNA has not yet been modified by DNA adenine methylase (the *dam* gene product). One protein is SeqA, which binds to the left half of *oriC* with tenfold greater affinity when this DNA is hemimethylated compared to when it is fully methylated. Footprinting experiments showed that SeqA specifically recognizes the hemimethylated GATC sequence in each 13-mer motif of *oriC* (Figure 1). These observations support a model in which SeqA blocks reinitiation by inhibiting the unwinding of the 13-mer region of *oriC*. In addition, SeqA seems to limit the binding of DnaA to *oriC*.

In *E. coli* with a generation time of 30 min, methylation of *oriC* after its duplication is delayed for ~13 min (~one-third of the cell cycle). In *seqA* mutants, methylation occurs after 5 min, so the 8 min difference is apparently because SeqA protein complexed to hemimethylated *oriC* impedes methylation. However, the 5 min delay indicates the participation of other factors. One of these is SeqB, a membrane-associated protein which appears to act in concert with SeqA. SeqB in a membrane fraction stimulates the binding of SeqA to hemimethylated *oriC* by at least 30-fold in DNA binding assays.

## Interaction of DnaA with Hda and the β-Subunit of DNA Polymerase III Holoenzyme

DnaA complexed to ATP is more active than the DnaA–ADP complex in initiation and unwinding of *oriC*, leading to the notion that the nucleotide bound state of DnaA controls initiation. Although DnaA alone can slowly hydrolyze ATP, hydrolysis is stimulated by

incubation with a recently discovered protein named Hda (for homologous to DnaA, specifically domain III), duplex or single-stranded DNA, and the $\beta$-subunit of DNA polymerase III holoenzyme. The $\beta$-subunit confers processive DNA synthesis to DNA polymerase III holoenzyme by tethering the enzyme to DNA during DNA synthesis. Because any duplex or single-stranded DNA suffices, ATP hydrolysis is not necessarily coupled to DNA replication. However, under appropriate *in vitro* conditions, the ATP bound to DnaA can by hydrolyzed by incubation with the $\beta$-subunit and Hda in coordination with DNA replication.

Hda protein is similar at the amino acid level to domain III of DnaA, and carries $AAA^+$ sequence motifs. Initially, the *hda* gene was described to be essential for viability. More recent experiments showed that viability, growth rate, initiation frequency, and replication synchrony were unaffected when a drug resistance gene interrupted the *hda* coding region. However, deletion of *hda* coding sequences caused asynchronous initiation. A model has been proposed that the interaction of DnaA with Hda and the $\beta$-subunit of DNA polymerase III holoenzyme, in coordination with DNA replication, stimulate the hydrolysis of ATP bound to DnaA to modulate the frequency of initiation. If so, the capacity for initiation after one round of initiation should diminish, reflecting the inactivation of DnaA function by ATP hydrolysis. However in the absence of sequestration, the initiation activity of DnaA was not diminished but was elevated instead. This suggests that the hydrolysis of ATP bound to DnaA does not control initiation frequency.

## Summary and Perspectives

Studies of the process of initiation from *oriC* has led to a molecular understanding of the events that lead to assembly of the replication fork machinery. Determination of the biochemical mechanisms that control this process will reveal how DNA replication occurs only once per cell cycle.

## See Also the Following Articles

Cell Cycle: Control of Entry and Progression Through S Phase • DNA Polymerase III, Bacterial • DNA Replication Fork, Bacterial

## Glossary

**DnaA box** A 9 bp DNA sequence motif recognized by DnaA protein.

**DnaA protein** The initiator of DNA replication, recognizing the chromosomal replication origin by binding to DnaA box sequences.

**DnaB protein** The replicative helicase that functions to unwind the parental duplex. Composed of six identical subunits, the single-stranded DNA passes through the central cavity of this ring-shaped protein when it unwinds double-stranded DNA.

**DNA polymerase III holoenzyme** The replicative DNA polymerase.

**DNA replication** The process of duplication of DNA.

***oriC*** The *Escherichia coli* chromosomal replication origin where DNA replication is initiated by recognition of DnaA box sequences.

**primase** The enzyme that forms primers needed for DNA replication by DNA polymerase III holoenzyme.

## Further Reading

Baker, T. A., and Bell, S. P. (1998). Polymerases and the replisome: Machines within machines. *Cell* **92**, 295–305.

Donachie, W. D., and Blakely, G. W. (2003). Coupling the initiation of chromosome replication to cell size in *Escherichia coli*.

Kornberg, A., and Baker, T. A. (1992). *DNA Replication*, 2nd edition. W.H. Freeman, New York.

Neidhardt, F. C., Ingraham, J. L., and Schaechter, M. (1990). *Physiology of the Bacterial Cell: A Molecular Approach*. Sinauer Associates, Sunderland, MA.

Nelson, D. L., and Cox, M. M. (2000). *Lehninger Principles of Biochemistry*, 3rd edition. Worth Publishers, New York.

## Biography

Jon M. Kaguni is a Professor in the Department of Biochemistry and Molecular Biology at Michigan State University. His principal research interests are on the mechanisms of DNA replication and its regulation. He holds a Ph.D. from the University of California, Los Angeles and received his postdoctoral training at the Stanford University School of Medicine. He developed the enzymatic system to study DNA replication from the *Escherichia coli* replication origin, and has studied the process of initiation of DNA replication throughout his professional career.

# DNA Restriction and Modification: Type I Enzymes

**David T. F. Dryden**
*University of Edinburgh, Edinburgh, UK*

**The original genetic studies of type I DNA restriction and modification (R/M) enzymes were published in 1953 by Bertani and Weigle, the same year that Watson and Crick presented their structural model of B-form DNA. Bertani and Weigle showed that some strains of *Escherichia coli* could reduce (restrict) the ability of bacteriophage to propagate through the bacterial population. Arber and colleagues later showed that surviving phage acquired a modification specific to the host bacterial strain, a modification which was lost upon subsequent passage through different hosts. The biological results were eventually linked to an enzyme-specific modification of DNA by type I R/M enzymes. These enzymes were designated *Type I* because they were the first restriction enzymes to be purified and characterized biochemically by Meselson and Yuan. The type II restriction enzymes prepared some years later had the very desirable property of cutting DNA into defined pieces, and they became the backbone of the genetic engineering revolution. In contrast, the type I enzymes, which cut DNA into unpredictable pieces, have provided important molecular models for complex biological mechanisms.**

## Biochemical Function

Type I restriction enzymes are large complexes with multiple enzymatic functions, and they possess the ability to switch between these functions depending upon the chemical nature of specific nucleotides within a target DNA sequence. A demonstrated function of type I enzymes in their natural hosts, eubacteria and archaea, is to protect the host from infection by foreign DNA from bacteriophage. This is achieved by two processes; modification of the host DNA by methylation at the N6 position of adenine nucleotides within the recognition sequence targeted by the enzyme and destruction of invading DNA, which typically lacks the appropriate modification of the target sequence. For a host specifying a type I restriction enzyme, typically far fewer than one in one thousand phage successfully infect the host. Therefore, they are a very effective defence system despite the considerable resources devoted by the host cell to their synthesis. This protective function is termed *restriction* and results in the destruction of the phage DNA once it has entered the host cell. Methylation of host DNA occurs during each round of DNA replication. The newly synthesized DNA strand lacks the appropriate methylation; the type I restriction enzyme recognizes this DNA as hemimethylated and adds the methyl group to the adenine in the target sequence in the newly synthesized strand. In this way, the type I restriction enzyme recognizes three different states of methylation of its target sequence, unmodified, hemimethylated, and fully modified, and performs a different reaction on each form of DNA, Figure 1. *In vitro*, the efficiency or accuracy of the switching between activities varies depending upon the particular type I restriction enzyme. For example, EcoKI, the type I restriction enzyme in *E. coli* K12 and EcoR124I, a plasmid encoded type I R/M system, are very precise—methylating only hemimethylated targets and cutting only unmodified ones. In contrast, EcoAI from *E. coli* 15T$^-$ methylates unmodified targets as effectively as hemimethylated targets, and, as a result, this activity competes with the restriction of unmodified DNA. These three enzymes are the best characterized examples of the type I restriction enzymes.

## Genes and Protein Structure

It has been shown that three genes are required to specify a type I system, *hsdS*, *hsdM*, and *hsdR*, where *hsd* refers to host specificity for DNA and *S*, *M*, and *R* stand for specificity, modification, and restriction, respectively. Typically, the *hsdR* gene is expressed from its own promoter and the *hsdM* and *hsdS* genes are expressed from a single promoter upstream of *hsdM*. *hsdR* is typically close to *hsdM/hsdS*, although some genome sequences suggest that this is not always the case. Genome rearrangements also occur around the *hsd* locus and this is used to induce some interesting switching of the expression and target sequence specificity of alternative type I R/M systems within a cell. Most studies

 © 2004, Elsevier Inc. All Rights Reserved. 

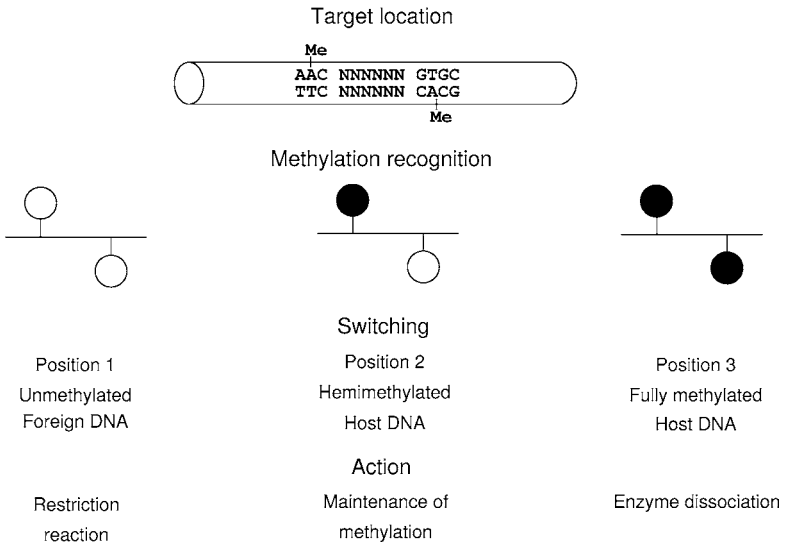



**FIGURE 1** The operations performed by a typical type I restriction enzyme are illustrated using the EcoKI enzyme binding to its DNA target sequence AACNNNNNNGTGC and determining the methylation status of the target. The sequence of operations is target location, methylation recognition, switching between different activities, and performance of the selected action.

have concentrated upon the type I systems of *E. coli* and *Salmonella enterica*, and it has been found in these hosts that type I R/M systems can be divided into at least four families based upon the following criteria: DNA hybridization, antibody cross reactivity, and genetic complementation of the *hsd* genes from different systems. These families are known as types IA, IB, IC, and ID. Structural and bioinformatics studies of the Hsd subunits have been used to understand the basis of these families. Within a family, amino acid sequence identity of the three expressed polypeptides is very high except for two regions of HsdS involved in DNA target recognition. Amino acid sequence identity between polypeptides from different families is much lower and is usually confined to regions of HsdS involved in DNA target recognition if the R/M systems being compared share at least part of the same DNA target sequence specificity, and to short motifs involved in catalysis.

## The Sequence Specificity Subunit

HsdS, often referred to as the S subunit, is responsible for recognizing the characteristic bipartite, asymmetric DNA target sequence of type I restriction enzymes, for example, the target for EcoKI is 5′-AACNNNNNNGTGC-3′, where N is any base. S subunits have a mass of approximately 50000 and comprise five regions of primary structure (polypeptide sequence), Figure 2. There are sequences (conserved regions) at the N terminus, the central part of the polypeptide, and at the C terminus that are highly conserved within a family of type I restriction enzymes and show some identity between families. Separating these three conserved regions are two longer sequences that display very limited sequence identity between different S subunits unless the different S subunits recognize the same DNA target sequence or one part of the same target sequence. These polypeptide sequences (variable regions) are associated with recognition of DNA target sequence and each region recognizes one part of the bipartite target sequence of the enzyme. These two variable regions each fold into a tertiary structure domain referred to as a target recognition domain (TRD). A weak sequence identity exists between the TRDs of S subunits and the TRD of methyltransferases from type II R/M systems. This identity covers a region involved in DNA sequence

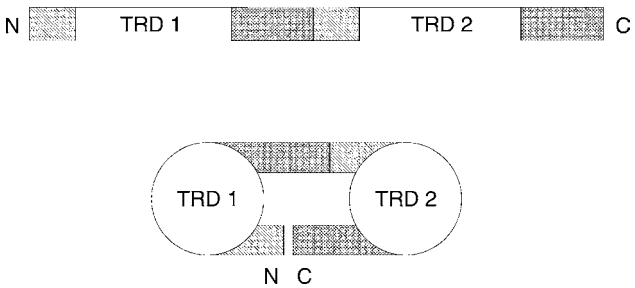



**FIGURE 2** Diagrams of a typical S subunit of a type I restriction enzyme. The upper diagram shows the subunit as a linear representation from the N terminus to the C terminus. Regions of sequence conservation between different S subunits are shown hatched. Regions within the subunit with sequence conservation have the same sort of hatching. The conserved regions flank the target recognition domains responsible for DNA sequence recognition. Each TRD recognizes one part of the bipartite target sequence. The lower diagram depicts the folded arrangement of these regions making apparent the approximate twofold rotational symmetry axis of the S subunits.

recognition, and extensive mutagenesis of a TRD from HsdS of EcoKI corroborated this similarity between TRDs from type I and type II R/M systems. The conserved polypeptide regions flanking the TRDs are structural components required to position the TRDs against the DNA target and to act as a scaffold for the binding of the M and R subunits. Some *hsdS* genes have been manipulated to exchange TRDs from different type I R/M systems. The resulting chimeric HsdS recognize new target DNA sequences. In addition, sequence similarity between conserved regions within an S subunit suggested an approximate twofold degree of symmetry in the subunit. A polypeptide comprising one TRD and part of the conserved regions formed a dimeric protein recognizing a twofold symmetrical DNA target. Within a family, the length of the central conserved region influences the number of nonspecific bases found in the DNA target sequence. Naturally occurring variation in the structure of *hsdS* genes has led to the evolution of new target specificities.

## The Modification Subunit

Two M subunits bind to HsdS to form a core trimeric enzyme with the properties of sequence specificity, detection of DNA methylation (whether hemimethylated or unmethylated), binding of methylation cofactor S-adenosyl-methionine (SAM), and methyltransferase activity. These M subunits are approximately 60000 molecular mass. The central one-third of the polypeptide sequence contains a series of amino acid motifs that are found in SAM-dependent methyltransferases and are involved in binding of the cofactor, binding of the adenine base specified for methylation, and catalytic transfer of the methyl group from SAM to the N6 position of the adenine base. This region of the M subunit has been successfully modeled upon the catalytic domain of the methyltransferases of type II R/M systems. Mutations in the motifs found in this catalytic domain have deleterious effects on activity. Methylation by type I restriction enzymes almost certainly occurs via the same nucleotide flipping mechanism used by methyltransferases of type II R/M systems in which the base targeted for modification is displaced from the DNA double helix into a catalytic pocket in the SAM-binding catalytic domain. The N terminal part of the M subunit of EcoKI appears to play a role in recognition of the methylation status of the DNA target sequence and mutations reduce the preference of EcoKI for methylating only hemimethylated DNA. The C terminal region of M subunits appears to play a role in interacting with the S subunit to form the core assembly. The polypeptide sequence of M subunits are almost identical within the same type I family but identity between families is mostly confined to the motifs in the catalytic domain.

## The Restriction Subunit

The complete type I restriction enzyme is formed by adding two R subunits to the core trimer to form a complex with subunit stoichiometry $R_2M_2S_1$ and a mass of around 440000, an enormous multifunctional molecular machine. As for HsdM, the R subunits show high sequence identity for members in the same family but lower identity, confined to a series of amino acid motifs, for comparisons between families. The complete restriction enzyme is still capable of methylating the DNA target sequence if it is hemimethylated and, if it is a type I system of low preference such as EcoAI, of methylating unmodified targets as well. However, unmethylated DNA target sequences primarily trigger the restriction reaction. The R subunits contain three main regions. An N terminal domain contains an amino acid motif found in DNA endonucleases particularly endonucleases from type II R/M systems. Mutagenesis experiments reveal that this domain is responsible for DNA cleavage by type I restriction enzymes. The central portion of the subunit contains a further set of amino acid motifs characteristic of the so-called DEAD-box helicases, enzymes which translocate or move DNA. These motifs form a multidomain structure that binds and hydrolyzes ATP and couples this hydrolysis to the movement of DNA relative to the enzyme (DNA translocation). Mutation abolishes the ability to hydrolyze ATP and translocate DNA. In the absence of translocation, the enzyme cannot cut DNA and restriction activity is lost even though the endonuclease domain is unaltered. The C terminal region of HsdR appears to be required for interaction with the core trimer.

## Assembly and Control of the Type I Restriction Enzyme

The assembly of the complete type I restriction enzyme is a complex affair and many type I R/M systems can form proteins with different subunit stoichiometries. The main partially assembled form of type I restriction enzymes is a trimer $M_2S_1$, which forms a fully functional modification methyltransferase recognizing the same DNA target as the complete enzyme. Other partially assembled forms and even individual subunits can be isolated and can display some limited activities such as sequence specificity, cofactor binding, and, for the $R_1M_2S_1$ form of EcoR124I, DNA methylation and ATP hydrolysis-driven DNA translocation. Intracellular concentrations of the Hsd subunits may vary with host conditions and it has been suggested that concentration-dependent assembly may play a role in the establishment of type I R/M systems in new hosts. It has also been shown that when the host cell acquires unmodified target sequences after suffering DNA

damage, the restriction activity of type IA and IB R/M systems is controlled by proteolytic digestion of hsdR. This control process prevents the type I restriction enzyme from attacking the unmodified target sequences in the damaged host chromosome.

## The Restriction Reaction

### DNA Binding and Recognition of Unmodified DNA

The type I restriction enzymes display a strong preference, in the presence of their cofactors, for binding to their DNA target sequence rather than to other DNA sequences. Although sequence specificity resides within HsdS, the presence of HsdM appears to be essential for strong binding to DNA. Once bound, the HsdM plus SAM detect the presence of adenine methylation on each strand of the DNA target using the base flipping mechanism. If the adenine is already methylated, then it will not be able to fit properly into the catalytic site as the methyl group will clash with the methyl group of SAM. This steric hindrance appears to change the conformation of the enzyme to allow the methylation reaction to proceed upon hemimethylated DNA or to dissociate from fully modified DNA. However, if both adenines are unmethylated, they can be accommodated within the HsdM along with SAM. The absence of steric hindrance for both target adenines appears to be the trigger for the highly complicated restriction reaction.

Upon recognition of an unmodified target site, the restriction enzyme becomes strongly attached to the DNA target site and does not appear to dissociate from the DNA after carrying out the restriction reaction. Strictly speaking, this lack of enzymatic turnover in the complete process means that type I restriction enzymes are not "enzymes." If the DNA molecule contains more than one target site, the type I restriction enzymes bound at each site can stick to each other to form a large complex. This additional DNA-dependent dimerization of the enzyme arises from diffusional motion of the DNA molecule bringing the bound enzymes into contact and does not depend upon enzyme activity. The functional role, if any, of this dimerization is not known, as it is not necessary for restriction.

### ATP Hydrolysis

Unmodified DNA target sequences trigger the hydrolysis of large amounts of ATP. The ATPase activity continues throughout the DNA translocation and cleavage process and even continues after DNA cleavage. The purpose of this continuing ATP hydrolysis observed *in vitro* is not known but may represent continuing DNA translocation on the cleaved DNA substrate.

### DNA Translocation

The enzyme remains bound to the target sequence and commences pulling DNA on either side of the target sequence toward itself. Because it is still attached at the target sequence, loops of DNA appear to be extruded from the enzyme, Figure 3. This process requires ATP hydrolysis and generates extensive supercoiling in the DNA. The reeling in of DNA occurs at approximately 100 to 400 base pairs per second and has been shown to occur over huge distances up to approximately 50000 base pairs. Stalling of the movement appears to occur when the translocating enzyme collides with a blockage on the DNA. This blockage can be another translocating type I restriction enzyme if there are two or more target sequences on a particular DNA molecule, stalling on a circular DNA molecule containing only one target sequence or collision with a complex DNA structure such as a fixed Holliday junction.

### Cleavage of DNA

Translocation blockage appears to be the signal for cutting of the DNA strands at the stalling location. Cutting does not occur at the original target sequence. As translocation rates of individual type I restriction enzymes on a DNA molecule may vary depending upon

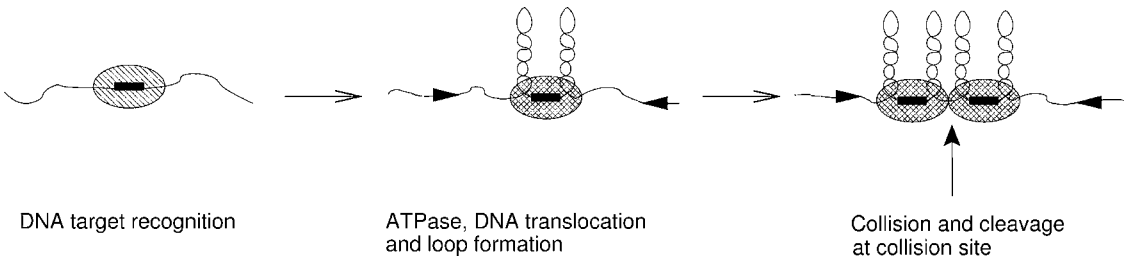



**FIGURE 3** The sequence of events occurring after the type I restriction enzyme (oval) has bound and recognized an unmodified DNA target sequence (black box) on a DNA molecule (thin line). ATP hydrolysis drives the translocation of DNA, reeling the DNA in toward the enzyme with, as a consequence, the extrusion of loops of DNA. Eventually, translocation is blocked by, in this example, collision with a second translocating type I restriction enzyme, whereupon cleavage occurs at the collision point. After cleavage *in vitro*, the enzymes remain bound to their target sequence.

the particular enzyme species and upon the sequence being translocated, individual enzymes will stall or collide with each other at different locations on the DNA. Therefore, in a solution containing many DNA molecules, translocation blockage and DNA cleavage occur at a range of locations. For a DNA molecule containing two or more target sequences for the same type I restriction enzyme, cleavage occurs approximately half way between the original DNA target sequences. For a DNA molecule containing target sequences for different type I restriction enzymes, the approximate location of cleavage depends on the relative translocation speeds of the different enzymes. DNA cleavage by type I restriction enzymes does not produce DNA fragments of defined length and sequence but rather a broad range of fragment lengths. Circular DNA molecules containing one target sequence are also cleaved at an undefined distance from the target sequence. The presence of a nick in a DNA molecule appears to bias the cleavage event to be close to the nick.

## Antirestriction

Despite their complexity, type I restriction enzymes are very effective barriers against bacteriophage propagation. Bacteriophage have developed a range of countermeasures to overcome this barrier. These antirestriction strategies include selection against presence of target sequences, modification of DNA bases, coinjection of bacteriophage proteins to inhibit restriction enzymes, hydrolysis of the SAM cofactor, and the use of genes encoding specialized proteins that act as competitive inhibitors of DNA binding by type I restriction enzymes. This last strategy is employed by bacteriophage T7, which produces gene 0.3 protein, also known as Ocr (overcome classical restriction), immediately upon infection of *E. coli*. This protein binds very strongly to all characterized type I restriction enzymes, irrespective of their DNA target sequences, and prevents the enzyme from binding to DNA. The structure of Ocr shows that negatively charged aspartate and glutamate side chains are arranged on the protein surface to perfectly match the three-dimensional positioning of the phosphate groups on DNA. Ocr appears to fit perfectly into the DNA-binding site of type I restriction enzymes and acts as a DNA mimic.

## See Also the Following Articles

DNA Restriction and Modification: Type II Enzymes • DNA Restriction and Modification: Type III Enzymes

## Glossary

**hsd** Host specificity of DNA. In a host expressing a type I R/M system, DNA containing the specificity target sequence becomes modified or restricted depending upon the methylation status of the target sequence.

**modification** The process accompanying restriction by which host DNA is maintained in a state of sequence-specific methylation. Most restriction enzymes cannot cleave DNA that has been modified by the methyltransferase partner. Type I restriction enzymes combine both restriction endonuclease and methyltransferase in one protein complex.

**restriction** The process of hindering the propagation of bacteriophage through a bacterial culture. This process requires cleavage of the bacteriophage DNA by DNA-sequence-specific endonucleases commonly referred to as restriction enzymes.

**translocation** Usually, the processive motion of an enzyme along DNA. However, for type I restriction enzymes which remain bound to their initial target sequence, refers to the pulling in of DNA toward the enzyme.

## Further Reading

Bertani, G., and Weigle, J. J. (1953). Host controlled variation in bacterial viruses. *J. Bacteriol.* **65**, 113–121.

Bickle, T. A., and Kruger, D. H. (1993). Biology of DNA Restriction. *Microbiol. Rev.* **57**, 434–450.

Burckhardt, J., Weisemann, J., Hamilton, D. L., and Yuan, R. (1981). Complexes formed between the restriction endonuclease EcoK and heteroduplex DNA. *J. Mol. Biol.* **153**, 425–440.

Davies, G. P., Martin, I., Sturrock, S. S., Cronshaw, A., Murray, N. E., and Dryden, D. T. F. (1999). On the structure and operation of type I DNA restriction enzymes. *J. Mol. Biol.* **290**, 565–579.

Davies, G. P., Kemp, P., Molineux, I. J., and Murray, N. E. (1999). The DNA translocation and ATPase activities of restriction-deficient mutants of EcoKI. *J. Mol. Biol.* **292**, 787–796.

Meselson, M., and Yuan, R. (1968). DNA restriction enzyme from *E. coli*. *Nature* **217**, 1110–1114.

Murray, N. E. (2000). Type I restriction systems: Sophisticated molecular machines (a legacy of Bertani and Weigle). *Microbiol. Mol. Biol. Rev.* **64**, 412–434.

Studier, F. W., and Bandyopadhyay, P. K. (1988). Model for how type I restriction enzymes select cleavage sites in DNA. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4677–4681.

Walkinshaw, M. D., Taylor, P., Sturrock, S. S., Atanasiu, C., Berge, T., Henderson, R. M., Edwardson, J. M., and Dryden, D. T. F. (2002). Structure of Ocr from bacteriophage T7, a protein that mimics B-form DNA. *Mol. Cell.* **9**, 187–194.

## Biography

David Dryden is a lecturer in physical chemistry at Edinburgh University, Scotland. He earned a degree in chemical physics and a Ph.D. in chemistry from Glasgow University. He gained postdoctoral experience with Roger Pain at Newcastle University. In 1989, he joined Noreen Murray at Edinburgh University to study type I DNA restriction enzymes. He started research on antirestriction proteins after being awarded a University Research Fellowship by the Royal Society in 1994. In 2002, he published, with Malcolm Walkinshaw (Edinburgh University), the first atomic structure of an antirestriction protein.

# DNA Restriction and Modification: Type II Enzymes

**Darren M. Gowers and Stephen E. Halford**
*University of Bristol, Bristol, UK*

**Type II restriction–modification (RM) enzymes are two separate proteins that act in concert to destroy foreign DNA as it enters a bacterial cell, while protecting the host DNA. Destruction of the foreign DNA is accomplished by a sequence-dependent restriction endonuclease, while a modification methyltransferase protects identical sequences in the host DNA. Restriction–modification systems are present in virtually all bacterial genera and show a huge range of DNA specificities. A complete listing can be found at REBASE (rebase.neb.com).**

## History

The ability of bacteria to defend themselves from viral infection was first reported by Luria and Bertani in 1952, who noted that some strains of *Escherichia coli* could restrict the growth of bacteriophage λ. This was accounted for a decade later by W. Arber, who proposed the existence of restriction–modification (RM) enzymes; the restriction enzymes were envisaged to cleave DNA in response to a particular sequence of bases, but only if the sites had not been protected by prior modification.

A landmark was reached in 1970, when H. O. Smith identified a novel restriction enzyme that cleaved DNA at a specific sequence. In collaboration with D. Nathans, he showed that this enzyme could be used to dissect DNA into discrete fragments. By 1978, when Arber, Nathans, and Smith shared the Nobel Prize in Medicine, nearly 50 restriction enzymes had been discovered. Today, that number stands near 3700. The early availability of restriction enzymes as off-the-shelf reagents heralded the age of recombinant DNA technology.

## Classification

At least three distinct categories of RM systems have been identified from their subunit compositions and their modes of action: types I, II, and III. This article focuses on the type II systems. In brief, most type II systems consist of two proteins: a restriction endonuclease that, in the presence of $Mg^{2+}$, cleaves DNA at a particular sequence, its recognition site; and a modification methyltransferase that blocks restriction activity by transferring a methyl group from S-adenosyl methionine (AdoMet) to a particular base in the recognition site. Only the nucleases from type II systems cleave DNA at specific positions and it is these, unlike the type I and the type III enzymes, which have the myriad applications in recombinant DNA technology.

The enzymes within the type II category display a variety of reaction mechanisms and subunit compositions. Many have been allotted to various subtypes, for example, the type IIS, IIB, IIE, and IIF enzymes.

## Discovery of New Restriction–Modification Systems

The value of type II restriction enzymes for DNA manipulations has driven extensive searches for more enzymes of this sort. A common means of identifying new restriction enzymes has been to screen bacterial lysates for endonucleases that cleave DNA into discrete fragments. Phage or adenoviral DNA are often used as test substrates. The recognition sites are then determined by sequencing methods. About one-half of the bacterial strains tested in this way are found to have a RM system, in some cases two or three such systems. But if the enzyme fails to cleave the test DNA for some reason, it will not be detected by this procedure.

New RM systems have also been identified by analyzing the genome sequences of bacteria. Such analyses show that almost all bacteria possess multiple candidates for RM genes, in some cases over 10 such candidates. Subsequent studies showed that a high proportion of the candidate genes were active RM systems, although some did not encode an active endonuclease.

 © 2004, Elsevier Inc. All Rights Reserved. 

## Organization of Genes

### Modification and Restriction Genes

The type II systems generally feature two separate genes: one for the restriction enzyme and one for the modification enzyme. In all such systems examined to date, the gene for the endonuclease is adjacent to that for the methyltransferase, although the order of the genes varies between systems. In some cases, they are in head-to-tail orientation and in others head-to-head or tail-to-tail.

Modification genes from different type II systems are often homologous to one another. In many cases, the methyltransferase genes encode 10 highly conserved blocks of amino acid sequence which are involved directly in catalysis and S-adenosyl methionine and AdoMet binding. Another block of protein sequence, which is not conserved, is involved in DNA recognition.

The restriction genes in the type II systems are never homologous to the modification gene from the same system, despite coding for proteins recognizing the same DNA sequence. Moreover, the restriction enzymes from different type II systems have dissimilar amino acid sequences, apart from enzymes with the same recognition site. The only well-conserved feature is the motif Pro-Asp-$X_{10-20}$-(Asp/Glu)-X-Lys (where X is any amino acid), which is often found at the active site. The aspartate and glutamate residues form part of the binding site for $Mg^{2+}$.

There are, however, many type II systems that deviate from the standard pattern of one modification gene and one restriction gene. For instance, some systems employ two modification enzymes, one for each strand of the DNA. Others have two restriction genes to encode an enzyme comprising two different subunits. In further cases, one gene encodes a single polypeptide with both modification and restriction activities.

### Regulating Restriction–Modification Expression

Whenever a bacteria gains a new RM system, expression of the RM genes has to be stringently coordinated. The methyltransferase must be produced first, to protect all the recognition sites in the chromosome, before significant amounts of the endonuclease are made. To do this, some type II RM systems, such as PvuII and BamHI, contain a gene upstream of the restriction gene that codes for a protein that regulates gene expression in this manner.

## The Type II Restriction Endonucleases

### Recognition Sequences

The recognition sequences for the majority of the type II restriction enzymes possess three attributes (Table I). First, the sites are usually 4–8 bp long. Second, the sites are palindromic: they read the same in 5′–3′ directions on both strands. Third, the sites are fully specified, in that every position in the sequence is occupied by a particular base. These enzymes cleave DNA at their recognition sites much more rapidly than any other sequence.

For some other enzymes, the recognition sequence is degenerate, in that certain positions can be occupied by alternative bases. For example, the recognition sequence for Cfr10I (Table I) has either G or A at its 5′-end and either C or T at its 3′-end. Similarly, the central base pair in the target site for EcoRII can be either an A:T or a T:A. For some systems, the recognition sequence is discontinuous, for example, SfiI (Table I). Such sites contain two blocks of specified sequence interrupted by a section of unspecified sequence but defined length that varies from just 1 bp (e.g., HinfI at GANTC) to 9 bp (e.g., XcmI at CCA$(N)_9$TGG). In most cases, the specified base pairs in the degenerate and in the discontinuous sites are still symmetrical palindromes.

Some recognition sites are truly asymmetric. For example, the 5′–3′ sequence in the top strand of the recognition site for BbvCI is not the same as the bottom strand (Table I). Many enzymes that recognize asymmetric sequences cleave the DNA some distance away from the site, at fixed positions on one side of the site. These are called the type IIS enzymes. One example is FokI at GGATG(9/13); it cleaves 9 bases downstream from its site on the top strand and 13 bases away on the bottom strand (Table I). However, some cut just one strand, for example, N.BstNBI (Table I). Conversely, some enzymes that recognize asymmetric sequences cleave both upstream *and* downstream of the recognition site, for example, BcgI (Table I). They cut four phosphodiester bonds in their reactions to liberate a small DNA fragment, 32 bp in the case of BcgI, which contains the intact recognition site. Such enzymes belong to the type IIB category.

The 3700 known enzymes have only 230 different recognition sequences, so enzymes from different species often have the same recognition site. Those that cut at exactly the same position are called isoschizomers; for example, HhaI and CfoI both cut GCG↓C. Those that cut at different positions within the same sequence are called neoschizomers; HinP1I, which cuts G↓CGC, is thus a neoschizomer of HhaI.

**TABLE I**

**Recognition Sites for Type II Restriction Endonucleases**

| Site | Enzyme | Genus | Recognition site |
|---|---|---|---|
| **Palindromic** | | | |
| **4 bp** | | | |
| 5′ overhangs | Sau3AI | *Staphylococcus aureus* | 5′ ↓ G-A-T-C 3′<br>3′ C-T-A-G ↑ 5′ |
| Blunt ends | AluI | *Arthrobacter luteus* | A-G ↓ C-T<br>T-C ↑ G-A |
| 3′ overhangs | HhaI | *Haemophilus haemolyticus* | G-C-G ↓ C<br>C ↑ G-C-G |
| **6 bp** | | | |
| 5′ overhangs | EcoRI | *Escherichia coli* | G ↓ A-A-T-T-C<br>C-T-T-A-A ↑ G |
| Blunt ends | EcoRV | *Escherichia coli* | G-A-T ↓ A-T-C<br>C-T-A ↑ T-A-G |
| 3′ overhangs | PvuI | *Proteus vulgaris* | C-G-A-T ↓ C-G<br>G-C ↑ T-A-G-C |
| **8 bp** | | | |
| 5′ overhangs | NotI | *Nocardia otitidis* | G-C ↓ G-G-C-C-G-C<br>C-G-C-C-G-G ↑ C-G |
| Blunt ends | PmeI | *Pseudomonas mendocina* | G-T-T-T ↓ A-A-A-C<br>C-A-A-A ↑ T-T-T-G |
| 3′ overhangs | SgfI | *Streptomyces griseoruber* | G-C-G-A-T ↓ C-G-C<br>C-G-C ↑ T-A-G-C-G |
| **Degenerate** | | | |
| | Cfr10I | *Citrobacter freundii* | (G/A) ↓ C-C-G-G-(C/T)<br>(C/T)-G-G-C-C ↑ (G/A) |
| | EcoRII | *Escherichia coli* | ↓ C-C-(A/T)-G-G<br>G-G-(T/A)-C-C ↑ |
| **Discontinuous** | | | |
| | SfiI | *Streptomyces fimbriatus* | G-G-C-C-$N_4$ ↓ N-G-G-C-C<br>C-C-G-G-N ↑ $N_4$-C-C-G-G |
| **Asymmetric** | | | |
| Cleaves within site | BbvCI | *Bacillus brevis* | C-C ↓ T-C-A-G-C<br>G-G-A-G-T ↑ C-G |
| Cleaves outside site | FokI | *Flavobacterium okeanokoites* | G-G-A-T-G-$N_9$ ↓ N-N-N-N<br>C-C-T-A-C-$N_9$-N-N-N-N ↑ |
| Cleaves one strand | N.BstNBI | *Bacillus stearothermophilus* | G-A-G-T-C-N-N-N-N ↓ N<br>C-T-C-A-G-N-N-N-N-N |
| Cleaves both sides | BcgI | *Bacillus coagulans* | ↓ $N_{10}$-C-G-A-$N_6$-T-G-C-$N_{12}$ ↓<br>↑ $N_{12}$-G-C-T-$N_6$-A-C-G-$N_{10}$ ↑ |

## Protein Structures

The enzymes that recognize palindromic DNA sequences are generally dimers of identical subunits. They interact with their recognition sequences symmetrically so that all the contacts between one subunit of the protein and one half of the recognition site are duplicated by the second subunit with the other half of the DNA. One active site in the dimer is positioned against the scissile phosphodiester bond in one strand of the DNA and likewise the second active site on the other strand.

The structures of several type II endonucleases, and their complexes with DNA, have been determined by X-ray crystallography. As with most other proteins that act at specific DNA sequences, restriction enzymes recognize their target sequences primarily by multiple hydrogen bonds to the bases in the major groove of the DNA. However, the restriction enzymes do not usually employ the elements of protein structure that are common amongst DNA-binding proteins, such as the helix–turn–helix or the zinc-finger. Instead, their DNA-recognition

elements encompass a wide variety of nonstandard structures, including loops, sheets, and helices. These also differ considerably from one enzyme to the next. In some cases, such as EcoRV, contacts to the specific sequence also depend on deformations to DNA structure.

Despite the differences in their structural elements for DNA recognition, and indeed in the overall dissimilarity in their amino acid sequences, some type II restriction enzymes have similar tertiary structures, for example, EcoRI and BamHI or EcoRV and PvuII. Similar structures have since been found in many other proteins that act on DNA, including enzymes involved in replication, recombination, repair, and transposition.

## Cleavage of Specific DNA Sequences

Enzymes such as EcoRI or EcoRV find their recognition sites in long DNA molecules by first binding to the DNA anywhere along the chain and then translocating to the recognition site. The transfer seems to occur primarily by multiple rounds of dissociation and reassociation events with the same chain. However, one-dimensional diffusion along the chain may also play a role, particularly over short distances of DNA. Once at the recognition site, each active site in the dimeric enzyme catalyzes the hydrolysis of its target phosphodiester bond in independent reactions. Both reactions are usually complete within the lifetime of the DNA–protein complex, so the initial product released from the enzyme is the DNA cut in both strands. However, if the complex dissociates before both strands are cut, for example, in reactions at high ionic strength, DNA cleaved in one strand is liberated.

With very few exceptions, the type II endonucleases require $Mg^{2+}$ for their reactions. Some other metal ions, such as $Mn^{2+}$ or $Co^{2+}$, give low levels of activity but others, such as $Ca^{2+}$, give no activity at all. The $Mg^{2+}$ ions play direct roles in the catalytic reaction, but the precise roles vary from enzyme to enzyme. Some, such as EcoRI and BglII, seem to have one metal ion at each active site, whereas EcoRV, BamHI, and many others use two ions.

The hydrolysis of phosphodiester bonds by type II endonucleases yields termini with 5′-phosphate and 3′-hydroxyl groups. Some enzymes introduce staggered breaks into the DNA, leaving fragments of double-stranded DNA with single-strand extensions at either their 5′-ends (e.g., EcoRI in Table I) or their 3′end (e.g., PvuI in Table I). Others cut both strands at equivalent positions, producing blunt-ended fragments (e.g., EcoRV in Table I).

## Interactions with Nonspecific DNA

The type II restriction enzymes are extremely specific for their cognate sequences. They cleave their recognition sites over a million times more rapidly than DNA sequences that differ from the recognition site by just 1 bp. Surprisingly, this specificity in catalysis need not be accompanied by an equivalent specificity in DNA binding. In the absence of $Mg^{2+}$ ions, many restriction enzymes bind to all DNA sequences with equal affinities. Their extraordinary specificities for DNA cleavage arise instead from the catalytic reaction, with only the cognate DNA giving rise to a catalytically competent enzyme–$Mg^{2+}$–DNA complex. For example, the complex of EcoRV with nonspecific DNA has a low affinity for $Mg^{2+}$, whereas its complex with specific DNA has a high affinity for $Mg^{2+}$, so only the specific DNA is cleaved.

Certain reaction conditions can, however, enhance the activities of these enzymes at alternative sites. This is termed star activity. The star sites are generally sequences that differ from the recognition site by 1 bp; sequences differing in two or more positions are normally resistant even under star conditions. The conditions that cause star activity include high pH, low ionic strength, the addition of organic solvents, and the replacement of $Mg^{2+}$ with $Mn^{2+}$.

## Subsets of Type II Restriction Enzymes

In recent years, many type II enzymes have been found to have distinctive properties and have been classified into subsets. Most of these enzymes have to interact with two copies of their recognition site before cleaving DNA. The restriction enzymes that need two sites are in effect double-checking the DNA to ensure that they cleave DNA only at the correct sequence.

### Type IIS

Type IIS enzymes recognize asymmetric sequences and cleave the DNA short distances downstream of the recognition site, typically 1–20 bp away. FokI (Table I) is the archetype of the type IIS enzymes. It is a monomeric protein containing two domains connected by a flexible linker: a DNA recognition domain that makes all of the contacts to the recognition site and a catalytic domain that has an active site capable of cleaving one DNA strand. To cut both strands, two monomers of FokI have to aggregate to a dimer via their catalytic domains to create a unit with two active sites. The dimer is formed most readily when both monomers are bound to separate FokI sites in the same DNA molecule. FokI is thus more active on molecules with two sites than on DNA with one site. This is a common feature of the type IIS enzymes. Most of them cleave DNA with two target sites more rapidly than DNA with one site even though they are not all monomers. Instead,

some are dimers, such as BfiI at ACTGGG(5/4), and others are tetramers, such as BspMI at ACCTGC(4/8).

### Type IIE

Another subset of type II enzymes is the type IIE group, typified by EcoRII and NaeI, which consists of dimers of identical subunits. In these enzymes, the interface between the subunits has two binding clefts for the recognition sequence rather than the single cleft in enzymes such as EcoRV and BamHI. One cleft has the catalytic functions for DNA cleavage, but these are inactive unless the other cleft has bound another copy of the recognition site. Interactions spanning two DNA sites occur more readily with sites in *cis* on the same molecule of DNA than with sites in *trans* on different molecules. The type IIE enzymes thus cleave DNA with two recognition sites faster than DNA with one site, but only one of the two sites is cut per turnover.

### Type IIF

SfiI, Cfr10I, NgoMIV, and several other restriction enzymes show further differences from the orthodox pattern, and this group is now known as the type IIF enzymes. They are tetramers of identical subunits, two of which interact with one copy of a palindromic recognition sequence while the other two interact with a second copy. To cleave DNA, both binding sites must be filled with cognate DNA. Hence, like the type IIE enzymes, the type IIF enzymes also interact with two copies of the target sequence, but, in contrast to the IIE systems, the IIF enzymes cut both sites in both strands. They thus cleave four phosphodiester bonds per turnover to convert a DNA with two sites into the product cut at both sites. They display optimal activity with sites in *cis* – the concurrent binding of the enzyme to sites in the same molecule of DNA traps the intervening DNA in a loop. Nevertheless, they can also act, albeit less efficiently, with sites in *trans* by bridging separate molecules.

### Type IIB

By cutting both strands of the DNA on both sides of their recognition sites, the type IIB enzymes such as BcgI (Table I) cut four phosphodiester bonds at *each* recognition site. Moreover, like many other restriction enzymes, the IIB enzymes are more active against DNA with two recognition sites than DNA with a single site. They thus have the potential to cleave eight phosphodiester bonds per turnover. The IIB enzymes also differ from the orthodox enzymes in subunit composition. For instance, BcgI contains two different subunits: one carries both the endonuclease and methyltransferase activities and the other recognizes the cognate DNA. Other type IIB enzymes contain a single subunit with endonuclease, methyltransferase, and DNA recognition functions. Moreover, some require AdoMet for not only the methyltransferase but also the endonuclease activity.

## The Type II Modification Methyltransferases

### Purpose of Methylation

The methyltransferases of type II RM systems are altogether different from the partner endonucleases; their only commonality is recognition of the same DNA sequence. The purpose of the modification enzyme is to protect recognition sites in the host DNA from the restriction enzyme. They achieve this by transferring a methyl group from AdoMet to a particular base in the recognition sequence. Some methylate the carbon at position 5 (C5) of the cytosine ring, some the exocyclic amino group at position 4 in cytosine, and some the exocyclic amino group at position 6 in adenine. Each turnover of a modification enzyme methylates the recognition sequence in one strand. This is sufficient to block the activity of the restriction endonuclease. Hence, following semiconservative replication of fully methylated DNA, the hemimethylated daughter strands are protected. The unmethylated strand must then be modified by the methyltransferase before the next round of replication.

### The Methyltransferase Enzymes

The methyltransferases from type II RM systems are monomeric proteins. The same enzyme methylates both strands of palindromic sites, but two methyltransferases are needed at asymmetric sites: one for the top strand and another for the bottom strand, although both activities are sometimes present in one polypeptide. The crystal structures of these enzymes have revealed two domains, one on either side of a DNA-binding cleft. One domain contains the DNA-recognition functions, which are unique to each methyltransferase, and the other the binding site for AdoMet and the catalytic residues for the transfer reaction.

The crystal structure of the HhaI methyltransferase bound to its recognition sequence revealed a novel perturbation of the DNA. Remarkably, the target cytidine had been swiveled completely out of its stacked conformation in the DNA helix and into the active site of the enzyme, deep in the catalytic domain. Other DNA-processing enzymes also use this mechanism, for example, the uracil DNA glycosidases in DNA repair. Base flipping may be a ubiquitous device used by enzymes that require access to the nucleotide bases in DNA that are otherwise buried in the double helix.

All methyltransferases use AdoMet as the methyl donor. With the cytosine C5 methyltransferases, the

carbon must first be activated. This is achieved by forming a covalent bond between the adjacent C6 position on the cytosine and the thiol group of a cysteine in the active site of the protein. The subsequent transfer of the methyl group to C5 expels the cysteine from C6, thus breaking the covalent link between protein and DNA. With the cytosine N4 and the adenine N6 methyltransferases, the exocyclic amino group may function directly as a nucleophile to attack the transferable methyl group of AdoMet.

## Restriction Enzymes in the Laboratory

Various factors have contributed to the adoption of type II restriction enzymes as essential research tools. The foremost is their specificity in cleaving DNA at fixed positions at particular sequences, that is, their abilities to recognize simple DNA sequences within any DNA molecule and to cleave the DNA at those sites without cleaving other sites to any detectable extent. Consequently, a restriction digest yields a discrete set of defined fragments determined by the positions of the recognition sites in the DNA molecule. Moreover, the resultant fragments of duplex DNA may, depending on the restriction enzyme being used (Table I), possess single-strand extensions that are mutually complementary. Such fragments can be then ligated to other DNA molecules with the same single-strand extensions.

The applications of restriction enzymes have led to the emergence of several companies for whom restriction enzymes are the primary product line. The availability of these enzymes permitted far-reaching technical advances, such as the construction of recombinant DNA molecules, restriction mapping, DNA sequencing, and the use of restriction-fragment-length polymorphisms (RFLPs) as genetic markers for genealogical analysis and gene isolation.

One feature of type II nucleases that concerns users is their star activity (noted previously). Under altered conditions, the barriers that prevent the enzymes from cleaving DNA at noncognate sites are less severe than those under optimal conditions. There is no general remedy for this, other than avoiding the conditions that cause it: low ionic strength, high pH, organic solvents, and $Mn^{2+}$. A further concern is the fact that many restriction enzymes cleave DNA only after interacting with two recognition sites. Such enzymes have low activities against DNA molecules with one target site, but they can be activated against single-site substrates by adding oligonucleotide duplexes that have the recognition sequence.

## See Also the Following Articles

DNA Restriction and Modification: Type I Enzymes • DNA Restriction and Modification: Type III Enzymes

## Glossary

**S-adenosylmethionine (AdoMet)** The cofactor for methyltransferases.

**palindromic DNA** A DNA in which the $5'-3'$ sequence of one strand is the same as that of the complementary strand.

**recognition site** A sequence of nucleotide bases recognized by a restriction enzyme.

**restriction digest** The cleavage of DNA molecules into defined fragments by the action of a (type II) restriction enzyme.

**restriction mapping** Placing the series of DNA fragments from a restriction digest into the order in which they occur along the DNA.

**restriction–modification (RM)** The bipartite bacterial defense system. Unmethylated phage DNA is hydrolyzed at specific sites by a *R*estriction enzyme; host DNA containing the same sequences is guarded from destruction by methylation from a *M*odification methyltransferase.

## Further Reading

Cheng, X., and Roberts, R. J. (2001). AdoMet-dependent methylation, DNA methyltransferases and base flipping. *Nucleic Acids Res.* **29**, 3784–3795.

Halford, S. E. (2001). Hopping, jumping and looping by restriction enzymes. *Biochem. Soc. Trans.* **29**, 363–373.

Pingoud, A., and Jeltsch, A. (2001). Structure and function of type-II restriction endonucleases. *Nucleic Acids Res.* **29**, 3705–3727.

REBASE. Available at: http://rebase.neb.com/rebase.

Roberts, R. J., and Macelis, D. (2001). REBASE-restriction enzymes and methylases. *Nucleic Acids Res.*, **29**, 268–269.

Roberts, R. J., Belfort, M., Bestor, T., Bhagwat, A. S., Bickle, T. A., Bitinaite, J., Blumenthal, R. M., Degtyarev, S. K., Dryden, D. T. F., Dybvig, K., Firman, K., Gromova, E. S., Gumport, R. I., Halford, S. E., Hattman, S., Heitman, J., Hornby, D. P., Janulaitis, A., Jeltsch, A., Josephsen, J., Kiss, A., Klaenhammer, T. R., Kobayashi, I., Kong, H., Kruger, D. H., Lacks, S., Marinus, M. G., Miyahara, M., Morgan, R. D., Murray, N. E., Nagaraja, V., Piekarowicz, A., Pingoud, A., Raleigh, E., Rao, D. N., Reich, N., Repin, V. E., Selker, E. U., Shaw, P-C., Stein, D. C., Stoddard, B. L., Szybalski, W., Trautner, T. A., Van Etten, J. L., Vitor, J. M. B., Wilson, G. G., and Xu, S-Y. (2003). A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res.*, in press.

## Biography

Darren Gowers is a postdoctoral researcher at Bristol University. His interests include the mechanisms of restriction enzymes that interact with two DNA sites, and the mechanisms by which sequence-specific DNA-binding proteins locate their target sequences. He gained his Ph.D. degree in 1998 from Southampton University.

Stephen Halford has led a research group at the University of Bristol since 1976, examining several different sorts of enzyme reactions on DNA, primarily the type II restriction endonucleases. He received his Ph.D. from Bristol University in 1970. In 2000, he was awarded the Novartis Medal of the Biochemical Society.

# DNA Restriction and Modification: Type III Enzymes

**Desirazu N. Rao and S. Srivani**
*Indian Institute of Science, Bangalore, India*

**Type III restriction–modification (RM) enzymes are multifunctional proteins that exhibit both restriction and modification activities. Five members in this class of enzymes have been characterized to some extent: *Eco*PI from prophage P1, *Eco*P15I from the prophage P1-related plasmid p15B in *Escherichia coli*, *Hin*fIII from *Haemophilus influenzae*, *Sty*LTI from *Salmonella typhimurium*, and *Lla*FI from *Lactococcus lactis*. These enzymes are composed of two subunits, products of the *res* and *mod* genes, and require ATP, S-adenosyl-L-methionine (AdoMet) and $Mg^{2+}$ for restriction. The Mod subunit alone functions as a methyltransferase in the presence of AdoMet, whereas restriction activity requires the cooperation of both the Res and Mod subunits. Type III restriction enzymes characteristically recognize DNA sequences that lack symmetry and cleave DNA 25–27 base pairs downstream of the sequence. Two inversely oriented (→←) unmethylated sites are the substrates for cleavage by type III restriction enzymes. It has been shown that ATP hydrolysis is required for DNA cleavage by the type III enzymes. In contrast, methylation proceeds regardless of the number and orientation of recognition sequences.**

## *Eco*PI and *Eco*P15I

*Eco*PI and *Eco*P15I restriction enzymes (R.*Eco*PI and R.*Eco*P15I) recognize the sequences 5′-AGACC-3′ and 5′-CAGCAG-3′, respectively. The *res* gene product is a 106-kDa protein, while the product of the *mod* gene is a 75-kDa protein. Analytical ultracentrifugation and gel quantification of *Eco*PI and *Eco*P15I restriction enzymes revealed a common $Res_2Mod_2$ subunit stoichiometry. The *Eco*PI and *Eco*P15I systems are closely related, in which complementation as well as recombination between the structural genes are possible. Electron microscopic analysis of heteroduplexes between the *Eco*P1 and *Eco*P15 genes reveals that the *res* genes of the two systems are largely homologous. The *mod* genes are a mosaic of homologous and nonhomologous regions, the latter being responsible for sequence specificity of these enzymes.

### Substrate Requirements

Cleavage of DNA by *Eco*P15I restriction enzyme (and the type III restriction enzymes in general) can be explained by a tracking–collision model (Figure 1). The model states that one *Eco*P15I molecule bound to a recognition site produces a DNA loop of increasing size as it tracks along the DNA until it collides with another *Eco*P15 molecule bound to another site tracking in the opposite direction. Translocation thus positions the two inversely oriented enzyme–site complexes appropriately for cleavage to occur. However, cleavage occurs at only one of the two possible cleavage positions and is a random event.

DNA translocation requires a driving force (ATP hydrolysis). It has been shown that the ATPase activity of *Eco*P15I and *Eco*PI is uniquely recognition site-specific. The collision complex is considered to be the endonucleolytically active form of the enzyme. Both cleavage products contain the original recognition sites, allowing the enzyme to remain bound and continue tracking. A DNA substrate possessing one *Eco*PI and one *Eco*P15I site in a head-to-head configuration was cleaved only in the presence of both enzymes, clearly demonstrating that these two different type III enzymes can functionally cooperate in DNA cleavage. Results from such cooperation assays using mutant enzymes suggest that double-strand breaks result from top strand cleavage by a Res subunit proximal to the site of cleavage, while bottom strand cleavage is catalyzed by a Res subunit supplied in trans by the distal endonuclease in the collision complex. DNA translocation appears not to be required for cleavage initiation, as two adjacent head-to-head or tail-to-tail oriented sites are efficiently cleaved. Post cleavage, the enzyme remains bound to the DNA. An exonuclease is required to act on these molecules releasing the enzyme for subsequent rounds of catalysis. Thus, efficiency of enzyme increases with decreased affinity for cleaved DNA.

One of the fundamental differences between type I and type III enzymes is that cleavage occurs at the point where the two DNA-translocating restriction enzyme complexes collide. While the cleavage by type III R–M

 © 2004, Elsevier Inc. All Rights Reserved. 

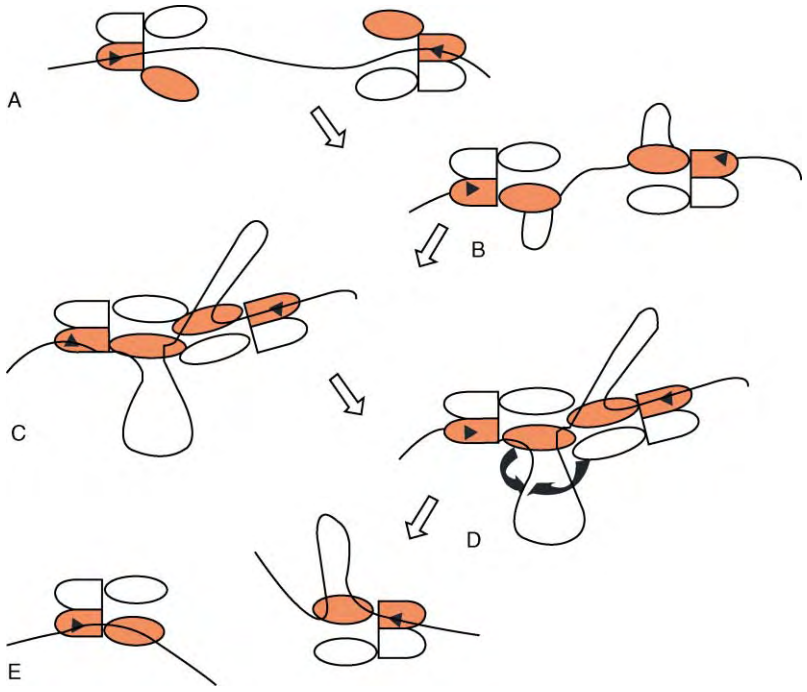



**FIGURE 1** Schematic diagram showing the model proposed for DNA cleavage by type III RM enzymes ( and represent Mod subunit; and represent Res subunit). (**A**) Enzyme binds two inversely oriented recognition sequences. (**B**) ATP-dependent DNA-translocation commences resulting in looping of DNA. (**C**) Collision of the two complexes results in conformational changes in Res subunit. (**D**) DNA cleavage: top strand is cut by the proximally attached Res subunit and bottom strand is cut by the distally attached Res subunit. (**E**) Cleaved DNA with the RM enzyme attached.

enzymes occurs 25–27 base pairs from the recognition sites, type I enzymes cleave 1000–7000 base pairs away from their recognition sites. Type III enzymes cleave at the recognition site proximal end of the tracking loop as illustrated in Figure 1. An unspecific molecular barrier such as a bound Lac-repressor, which would halt DNA translocation, is not adequate to evoke DNA cleavage by the type III enzyme, *Eco*P15I. Despite differences in structure and mode of action, type I and type III enzymes appear closely related mechanistically.

## Cofactor Requirements

Both *Eco*P15I and *Eco*PI restriction enzymes possess sequence-specific ATPase activity. Nonhydrolyzable analogs of ATP, such as AMP-PNP or ATP-γS, cannot replace ATP in the reaction. DNA restriction by *Eco*PI restriction enzyme requires exogenous addition of AdoMet, while the closely related *Eco*P15I enzyme has bound AdoMet and therefore, does not require the addition of this cofactor for DNA cleavage. Reconstitution experiments, which involve mixing of the isolated subunits of R.*Eco*P15I result in an apoenzyme form, which is restriction proficient only in the presence of AdoMet. However, mixing the Res subunit with Mod subunit deficient in AdoMet binding does not result in a functional restriction enzyme, suggesting that AdoMet binding causes a conformational change, which is necessary for DNA cleavage.

## Organization of Genes and Regulation of Restriction Activity

Several observations implied that the *mod–res* operon was transcribed as a single unit. Sequence analysis of the genes showed a two base pair gap between the end of *mod* and the beginning of *res* gene. This led to the conclusion that translation of the *res* gene was due to ribosomal shuffling from the terminator to the initiator codon, an initiation factor independent event.

A tight control of the potentially lethal activity of the restriction enzyme must be ensured for efficient establishment of type III restriction–modification (RM) system in a cell. Two independent post-transcriptional regulatory mechanisms control the expression of restriction activity: (a) The modification activity is expressed immediately after the RM genes enter the cell, whereas the expression of restriction activity is delayed until the complete protection of cellular DNA is achieved by methylation. The expression of the modification subunit regulates the amount of restriction subunit present in the cell, suggesting that the correct folding of Res into active and stable configuration is promoted by its interaction with the Mod subunit. (b) *In vivo* restriction activity is modulated by a decrease in the efficiency of translation and by varying ribosomal accuracy conditions.

### Domainal Organization in Restriction Subunit

Multiple sequence alignment of all known and putative Res subunits suggests a modular structure (Figure 2). The ATPase and helicase domains are present in the N-terminal region of Res subunit. The C-terminus contains the P-D…(D/E)-X-K motif that is commonly present in the catalytic center of type II restriction endonucleases. Amino acid sequence comparison of the restriction subunit of *Eco*PI and several putative Res subunits revealed the so-called DEAD box motif that is present in the helicase superfamily II. The members of the DEAD family have seven conserved motifs (motifs I, IA, II–VI). The first of the seven motifs of the DEAD family of helicases resembles the Walker A domain. Mutational analysis of motif I resulted in a loss of DNA cleavage and ATP hydrolysis, while that of motif II significantly decreased ATP hydrolysis but had no effect on DNA cleavage. These motifs must, therefore, clearly play a role in ATP hydrolysis. Mutations in motif VI abolished both activities while mutations in the putative endonuclease active site of Res P1 and Res P15 abolished DNA cleavage, but not ATP hydrolysis.

*Eco*PI and *Eco*P15I restriction enzymes are also modification methylases, more efficient than their methylase counterparts. Studies with *Eco*P15I enzyme revealed that the cofactor requirements for methylation by the modification methylase were different from those for the restriction enzyme. $Mg^{2+}$ is an absolute requirement for the modification methylase, while the restriction enzyme could methylate DNA in the absence of $Mg^{2+}$. Another contrasting feature is that while ATP stimulates methylation activity of the restriction enzyme, the activity of the modification methylase is not altered. It is thought that stimulation of methylation activity of *Eco*P15I restriction enzyme could be due to DNA tracking which transforms modification from a distributive to a processive reaction.

## *Hinf* III

*Hinf* III and *Hine*I restriction enzymes recognize the sequence 5′-CGAAT-3′. *Hinf* III enzyme exists as a large complex of two subunits with a molecular size greater than 200 kDa. Two forms of the enzyme, one with AdoMet bound and the other without the cofactor bound are known to occur. Both forms of the enzyme cleave DNA, only in the presence of ATP and $Mg^{2+}$, which is stimulated by AdoMet.

A search of the whole genome sequence of *H. influenzae* strain Rd identified tetra nucleotide repeats in a gene, with low homology to *Eco*PI *mod* gene. The adjacent downstream gene in the *H. influenzae* genome has low homology to the *res* gene of the type III family. This homology and organization is a strong indication that these genes indeed encode a type III restriction system. It is, therefore, likely that *Hinf* III purified from *H. influenzae* Rf strain is encoded by the *mod* and *res* genes described above. The number of repeats within the *mod* gene influences the rate of phase variation and expression of the *mod* gene. Similarly, a pentanucleotide repeat in the *mod* gene of *Pasteurella haemolytica* may modulate expression of a resident type III system.

## Other Type III RM Systems

With the sequencing of a number of genomes, a number of putative RM systems have been discovered. A few RM systems have been characterized but not in extensive detail such as those mentioned earlier.

The *res* and *mod* genes for *Sty*LTI system have been cloned and sequenced. The sequence 5′-CAGAG-3′ was found to be the canonical recognition sequence and methylation was limited to the second adenine in this sequence. Based on the derived amino acid sequence, the *Sty*LT system shows a high degree of homology with the other type III systems. *Lla*FI is the first type III RM system to be characterized from a Gram-positive bacterium, *Lactococcus lactis*. *Lla*FI has cofactor

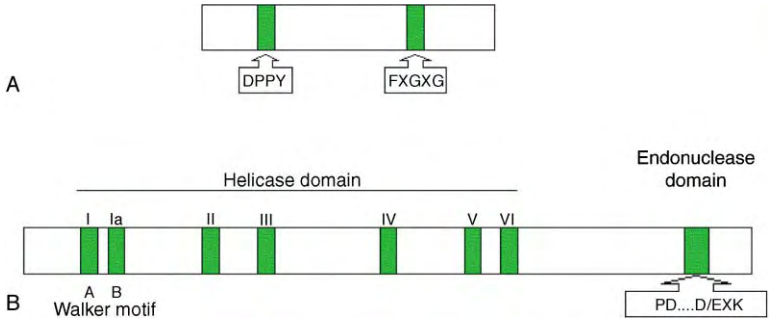



**FIGURE 2** Modular structure of type III restriction enzyme. (**A**) Mod subunit showing the location of conserved amino acid sequence regions; catalytic motif, DPPY (motif IV) and AdoMet binding motif, FXGXG (motif I). (**B**) Res subunit showing the location of conserved sequences, helicase motif, and putative endonuclease motif.

requirements that are characteristic of type III enzymes. ATP and $Mg^{2+}$ are required while AdoMet stimulates DNA cleavage. An open reading frame (ORF) from an 8 kb fragment from *Bacillus cereus* ATCC 10987 showed 29.3% identity with the *Eco*PI *res* gene product and 67.6% identity with the deduced amino acid sequence of *Sty*LTI system. Based on these observations, a type III system has been suggested to exist in *B. cereus*. The complete genome sequence of the gastric pathogen *Helicobacter pylori* has revealed four type III RM systems identified on the basis of gene order and similarity to endonucleases and methyltransferases.

## DNA Methyltransferases

One unique feature of methylation by *Eco*PI and other type III RM systems is that only one strand of the asymmetric recognition sequence is methylated. Thus, unmodified DNA is the only substrate for the reaction. *Eco*P15I DNA methyltransferase (*Eco*P15I MTase) adds a methyl group to the second adenine in the recognition sequence 5′-CAGCAG-3′ in the presence of AdoMet and $Mg^{2+}$. *Eco*P15I MTase occurs as a dimer in solution. The kinetic mechanism for *Eco*P15I MTase has been elucidated and the order of substrates binding is random. *Eco*P15I MTase binds about threefold more tightly to DNA containing its recognition sequence 5′-CAGCAG-3′ than to nonspecific sequences in the absence or presence of cofactors. In the presence of ATP, the discrimination between specific and nonspecific sequences increases significantly, suggesting a role for ATP in DNA recognition by these enzymes. *Eco*P15I DNA MTase makes contacts in the major groove of its substrate DNA. The first guanine of the recognition sequence does not show any altered reactivity toward dimethyl sulfate, but the second guanine was modified 3.5-fold better in the presence of the enzyme. This hypermethylation of guanine next to the target adenine could be due to a conformational change in the DNA as a result of protein binding. This probably indicates that the protein flips out the target adenine residue. Both potassium permanganate footprinting and fluorescence spectroscopic measurements support a base flipping mechanism for *Eco*P15I DNA MTase. *Eco*P15I MTase and *Eco*PI MTase, like other $N^6$-MTases, contain two highly conserved sequences, FxGxG (motif I) and DPPY (motif IV) postulated to form the AdoMet binding site and catalytic site, respectively. Mutations in motif I completely destroy AdoMet binding but leave target DNA recognition unaltered. Mutations in motif IV result in loss of enzyme activity but enhance crosslinking of AdoMet and DNA, implying that DNA and AdoMet binding sites are close to motif IV.

*Eco*PI DNA methyltransferase methylates the second adenine in the recognition sequence 5′-AGACC-3′. Replacement of tyrosine in the conserved DPPY motif with tryptophan results in a mutant enzyme that binds AdoMet almost as well as the wild-type but is catalytically inactive. However, DNA binding is at least threefold stronger than that of the wild-type enzyme.

Clear plaque mutants of phage P1 were isolated to those defective in DNA modification and map to the nonhomologous region of the *mod* gene of P1. These mutant methylases (*c*2 mutants) have either very little or no AdoMet binding and therefore have very weak or no methylating activity. However, the mutant proteins bind specifically to DNA containing *Eco*P1 recognition sequences.

## See Also the Following Articles

DNA Methyltransferases, Bacterial • DNA Restriction and Modification: Type I Enzymes • DNA Restriction and Modification: Type II Enzymes

## Glossary

**base flipping** Process in which a base in the normal B-DNA is swung out of the helix into an extrahelical position.

**DNA translocation** Movement of DNA through a region of protein, an energy-dependent process.

**methylation** Modification of target base within the recognition sequence by the transfer of a methyl group from AdoMet.

**restriction** DNA cleavage by a restriction enzyme within or outside the recognition site.

## Further Reading

Aude, A., Bourniquel, A., and Bickle, T. A. (2002). Complex restriction enzymes: NTP-driven molecular motors. *Biochimie* **84**, 1047–1059.

Dryden, D. T. F., Murray, N. E., and Rao, D. N. (2001). Nucleoside triphosphate-dependent restriction enzymes. *Nucleic Acids Res.* **29**, 3728–3741.

Krüger, D. H., Küpper, D., Meisel, A., Reüter, M., and Schroeder, C. (1995). The significance of distance and orientation of restriction endonuclease recognition sites in viral DNA genomes. *FEMS Microbiol. Rev.* **17**, 177–184.

Rao, D. N., Saha, S., and Krishnamurthy, V. (2000). ATP-dependent restriction enzymes. *Prog. Nucl. Acid Res. Mol. Biol.* **64**, 1–63.

## Biography

Desirazu N. Rao is a Professor in the Department of Biochemistry at the Indian Institute of Science, Bangalore, India. His principal research interests are in the area of DNA–protein interactions using restriction–modification enzymes and DNA mismatch repair proteins as model systems. He holds a Ph.D. from the Indian Institute of Science and received his postdoctoral training at the National Institutes of Health, Bethesda, Maryland; Biozentrum, University of Basel, Switzerland; and the University of Cambridge, United Kingdom.

S. Srivani is a Ph.D. student in the Biochemistry Department, Indian Institute of Science.

# DNA Secondary Structure

**Albino Bacolla and Robert D. Wells**
*Institute of Biosciences & Technology, Houston, Texas, USA*

**The term secondary structure refers to all DNA conformations (non-*B*-DNA) other than the antiparallel right-handed double helix (*B*-DNA). The conventional and commonly accepted structure of the majority of DNA in a cell is that of *B*-DNA, as first proposed by Watson and Crick in 1953. However, experimental data have shown for almost 50 years that nucleic acids exist in a variety of other conformations, including left-handed duplexes and three- and four-stranded helices, with parallel and antiparallel strand orientations. In addition, recent nuclear magnetic resonance (NMR) studies have revealed the assembly of nucleic acids into pentameric and hexameric architectures. These non-*B* conformations are favored by specific sequence motifs at certain chromosomal loci and by defined topological or environmental conditions, such as negative supercoil density or cations.**

## *B*-DNA

In 1980, the crystal structure of a helix turn of DNA was reported, which confirmed the Watson and Crick model. Since then, analyses of many sequences agree that the structure of DNA (*B*-DNA) is a right-handed double helix rotating about a central axis of symmetry and forming a major and a minor groove (Figure 1A). The Watson and Crick base-pairing scheme between a purine and pyrimidine base (adenine (A) with thymine (T) and cytosine (C) with guanine (G)) was confirmed. Deviations from these pairings (mismatches) perturb the helix structure and are readily corrected by the cellular DNA repair enzymes.

## *Z*-DNA

The first crystal structure of a DNA minihelix, reported in 1979, revealed a left-handed double helix. It was called *Z*-DNA because of the zigzag shape of the phosphate and sugar backbone.

### Structure

*Z*-DNA is formed by alternating purine–pyrimidine (RY·RY) sequences (where R indicates a purine, A or G, and Y indicates a pyrimidine, C or T; the dot designates the complementary strands), such as the repeating $(CG{\cdot}CG)_n$ and $(CA{\cdot}TG)_n$ motifs (Table I). Structurally, the *Z*-DNA helix is slimmer and more elongated than *B*-DNA, and it lacks a major groove (Figure 1B). *Z*-DNA represents a high-energy state for DNA; *in vivo*, most energy required for the *B* to *Z* transition is supplied by negative supercoiling, a topological state found in chromosomal DNA.

### Biology

Alternating (RY·RY) tracts are common in eukaryota, and *Z*-DNA formation has been found mostly in transcribed regions of the genome. In the current model, high negative supercoiling generated behind RNA polymerase complexes during transcription drives the (RY·RY) tracts into a left-handed conformation. The alternative structure, in turn, regulates gene expression. For example, the rat nucleolin promoter contains a $[(CA)_{10}(CG)_8]$ motif that represses promoter activity upon adopting a left-handed conformation. Allelic variants differing in the number of dinucleotide repeats show a quantitative effect on the rate of gene transcription that correlates with the stability of *Z*-DNA. Sera from patients with the autoimmune systemic disease *Lupus erythematosus* contain antibodies against *Z*-DNA. Two eukaryotic proteins, both $\gamma$-interferon-inducible, bind and stabilize *Z*-DNA structure: ADAR1, an RNA-editing enzyme, and DLM-1, a protein that is up-regulated in tumor stromal cells.

## Triplex DNA

The association of synthetic nucleic acids into three-stranded structures (three-stranded DNA or H-DNA) was reported in 1957 and was confirmed in 1968 with naturally occurring sequences.

### Structure

In a three-stranded nucleic acid, consecutive purine bases in a *B*-DNA duplex engage a third base through additional hydrogen bonds, referred to as Hoogsteen hydrogen bonds. These bonds differ from the Watson

 © 2004, Elsevier Inc. All Rights Reserved. 

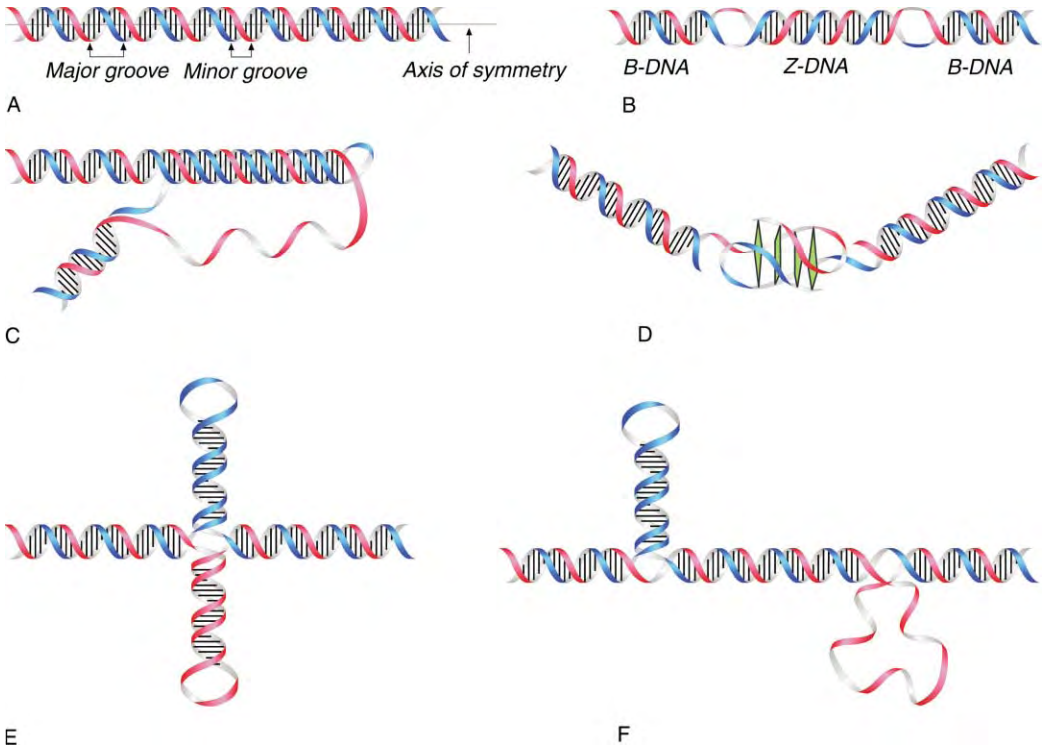



**FIGURE 1** DNA secondary structures. (A) *B*-DNA, a canonical antiparallel right-handed double helix with major and minor grooves. (B) *Z*-DNA, a left-handed double helix. The ribbons show the transition between right-handed and left-handed sections. (C) Triplex DNA; ribbon shows a third strand wrapping in the major groove and the complementary strand unpaired. (D) Tetraplex DNA; the model shows an intermolecular tetraplex containing antiparallel strands from two duplex molecules. (E) Cruciforms; the model shows a four-way junction and single-stranded loops. (F) Slipped DNA; the ribbons illustrate two conformationally different slipped structures, a base-paired hairpin and an unpaired loop. Reprinted from R. D. Wells and S. T. Warren, *Genetic Instabilities and Hereditary Neurological Diseases*, p. 563, Copyright 1998, with permission from Elsevier.

and Crick model and were described by Hoogsteen in 1963 when an A·T base pair was first reported. The formation of a triplex requires DNA to contain only purines in one strand and pyrimidines in the complementary strand (R·Y) in a mirror repeat orientation (Table I). In an intramolecular triplex, one-half of the purine-rich (or pyrimidine-rich) strand dissociates and folds back to engage the remaining Watson and Crick paired purine-rich strand in the major groove (Figure 1C). Alternatively, in an intermolecular triplex, a purine (or pyrimidine) strand from one DNA tract engages in Hoogsteen bonds with a duplex from a different DNA tract. Specific bonds form between A and A, G and G, A and T, and G and protonated C.

## Biology

R·Y tracts are common in eukaryotic genomes. In humans, sequences with thousands of such asymmetric base pairs exist, such as in the intron 21 of the

**TABLE I**

**DNA Secondary Structures, Sequence, Sequence Features, and Biology**

| Secondary structure | Sequence requirements | Examples | Biology |
|---|---|---|---|
| Left-handed DNA (*Z*-DNA) | $(RY{\cdot}RY)_n$ | $(CG{\cdot}CG)_{40}$<br>$[(CA)_{10}(CG)_8{\cdot}(CG)_8(TG)_{10}]$ | Gene expression |
| Triplex DNA (H-DNA) | $(R{\cdot}Y)_n$ (mirror repeats) | $(GAA{\cdot}TTC)_{18}$<br>$(GA{\cdot}TC)_n$ | Gene expression, chromatin organization, genetic instability |
| Tetraplex DNA (G4-DNA) | Repeating G-tracts | $(TTAGGG)_4$<br>$[(TGGGGAGGG)_2TGGGGAAGG]$ | Telomere capping, gene expression |
| Cruciforms | Inverted repeats | GTCCAGTATACTGGAC | Replication origins, genetic instability |
| Slipped DNA (hairpins) | Direct repeats | $(CTG{\cdot}CAG)_{180}$ | Gene expression, genetic instability |

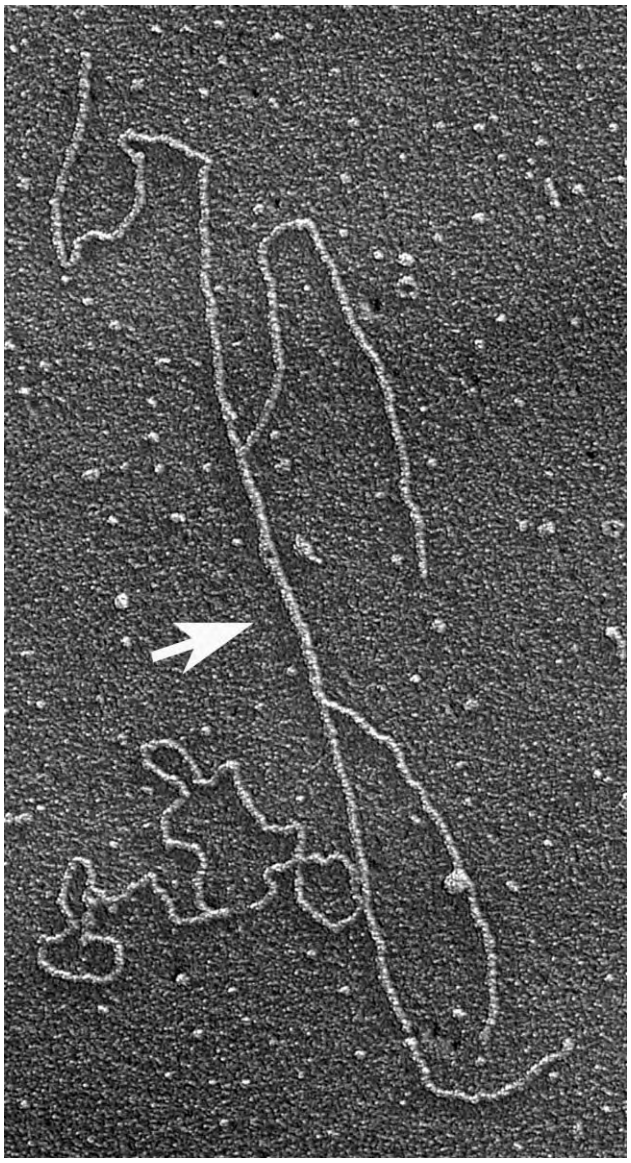


**FIGURE 2** Electron micrograph of sticky DNA. Two linear DNA molecules interact (arrow) and are held together by a pair of long GAA·TTC tracts from a Friedreich's ataxia patient. Courtesy of Dr. Jack Griffith of the University of North Carolina, Chapel Hill, NC.

polycystic kidney disease gene 1. Triplex structures have been shown to inhibit transcription and replication, but increase recombination rates and promote genome instability. Sticky DNA (Figure 2) is a long triplex between two tracts of $(GAA{\cdot}TTC)_n$ (where $n = 59-270$) that are distantly located in a circular DNA. Friedreich's ataxia (FRDA), the most common hereditary ataxia, is caused by the expansion of the GAA·TTC tract in intron 1 of the frataxin gene, which subsequently inhibits its transcription. Sticky DNA is likely to be involved in the etiology of this neurological disease. In mammalian nuclei, actively transcribed chromatin is organized in specific domains, and centromeres are involved in such organization. Visualization of triplexes in nuclei revealed a colocalization of such structures from centromeres and other loci, supporting a model in which triplexes are implicated in the organization of chromatin domains.

### Pharmacology

The delivery of synthetic triplex-forming oligonucleotides (TFOs) that adopt Hoogsteen pairs with complementary purine-rich genomic targets has been shown to form triplexes in nuclei. This strategy has been explored as a therapeutic tool to inhibit gene expression. For example, a TFO targeting an R·Y tract in the third intron of the *ICAM-1* gene, whose up-regulation is associated with inflammatory diseases, inhibited protein synthesis in human keratinocytes in cell culture and is considered a therapeutic agent in the treatment of psoriasis.

## Tetraplex DNA

The ability of G residues to assemble in tetrameric arrangements (quadruplex DNA, four-stranded DNA, or G4 DNA) was noted in 1962. In 1988, tetraplex DNA was reported in biological systems.

### Structure

Tetraplex DNA (also called G4 DNA because of the G quartets) forms when DNA sequences are composed of four repeating motifs that contain G residues (Figure 1D, Table I). Gs from each motif stack on one another and the four stacks form the sides of a cube. In each stack, four Gs hydrogen-bond and constitute a tetrad. NMR and X-ray crystallography studies showed that, in fact, all four nucleotides can form heterotetrads (or homotetrads), and therefore tetraplex DNA is likely to be observed with a greater variety of sequences *in vivo*.

### Biology

Tetraplex DNA is involved in the maintenance of telomere ends and in the control of gene expression.

#### Maintenance of Telomere Ends

The DNA sequence at the ends of eukaryotic linear chromosomes, the telomeres, is a repetitive sequence that contains tetraplex-forming Gs. It is postulated that such capping protects the chromosome ends from being degraded and mediates chromosome condensation. Several proteins assist in the formation of tetraplex DNA; others disassemble the structure.

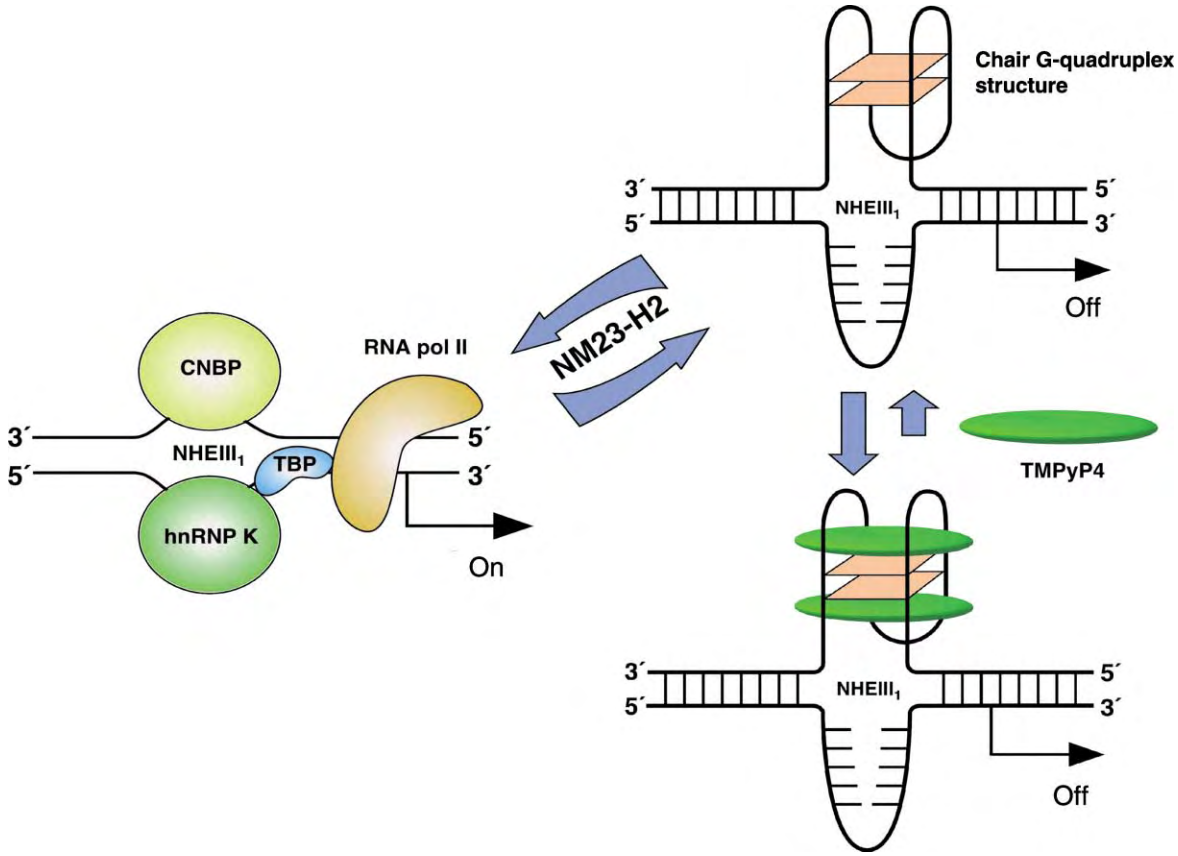



**FIGURE 3** Inhibition of transcription by tetraplex DNA, a model for the inhibition of transcription at the *c-MYC* promoter. Left, the transcriptional complex assembles at the promoter and supports initiation of transcription by an RNA polymerase II complex. Top right, the cellular factor NM23-H2 promotes the transition from duplex to tetraplex, preventing assembly of transcription factors. Bottom right, TMPyP4 binds to, and stabilizes, the tetraplex structure driving the equilibrium toward stable transcriptional repression. TMPyP4, 5,10,15,20-tetra(*N*-methyl-4-pyridyl)porphine, a porphyrin derivative, and pyrelene derivatives such as *N*,*N*′(-bis[2-(1-piperidino)-ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER) are explored as therapeutic agents because their tetraplex binding activity inhibits telomerase, which is up-regulated in 80–90% of malignant tumors. Nuclear hypersensitive element $III_1$ ($NHEIII_1$) is a 27-bp promoter sequence cleaved when probed with single-stranded-specific nucleases or other chemicals. This sequence is shown in Table I. Reprinted from Siddiqui-Jain, A., Grand, C. L., Bears, D. J., and Hurley, L. H. (2002). Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress a c-*MYC* transcription. *Proc. Natl Acad. Sci. USA* **99**, 11593–11598. Copyright 2002, with permission of National Academy of Sciences, U.S.A.

### Control of Gene Expression

Human sequences in the immunoglobulin switch region and in the promoters of other genes, including the oncogenes *c-MYC*, *c-MYB*, *c-FOS*, and *c-ABL*, contain repeating Gs that have been shown to form G-quartets. Experiments in cell cultures indicate that the promoter region of *c-MYC* is under the regulatory control of a secondary DNA structure, including a tetraplex. Specifically, its formation decreases transcriptional activity by interfering with the assembly of an RNA–polymerase complex (Figure 3).

## Cruciforms and Slipped Structures

Cruciforms and slipped structures are formed by repetitive DNA sequences, but do not require a specific base composition.

### Cruciforms

In inverted repeats, a DNA sequence is followed by its complementary sequence read in the opposite orientation (Table I). Thus, each strand may form a fully paired duplex, giving rise to a cruciform (Figure 1E). Inverted repeats are common at replication origins and at chromosomal locations associated with genomic instability.

### Origins of Replication

Bacteria may carry extra-chromosomal DNA elements called plasmids. pT181 belongs to a class of plasmids whose replication requires nicking on one strand; under optimal conditions, negative supercoiling drives the formation of a cruciform that positions the nicking site on a single-stranded loop, allowing

nicking and the replisome to assemble and carry out replication. Studies have indicated a dynamic distribution of cruciforms in mammalian nuclei, their number being maximal at the $G_1$/S phase boundary, when DNA synthesis takes place. The concentration of a family of proteins (called 14-3-3) with cruciform-binding activities is also maximal in this period. Experiments on monkey *ors8* and *ors12* replication origins support the involvement of such proteins and DNA secondary structures in the initiation of DNA replication.

### Genetic Instability

The recurrent human translocation t(11;22) involves the fusion of chromosomes 11 and 22. The site of translocation contains several hundred base pairs of AT-rich sequences on both chromosomes that may form large cruciforms. Breaking points were observed at the center of an inverted repeat sequence on both chromosomes, supporting the formation of a cruciform structure as the cause of translocation (Figure 4).

## Slipped DNA

Repeating motifs of identical sequence composition (direct repeats) may base-pair in an out-of-register manner and thus form looped-out (slipped) structures (Figure 1F). Di-, tri-, tetra-, and pentanucleotide repeats are common in prokaryotes and eukaryotes and are often present in regulatory regions. Their copy number varies within a population, and this variation modulates gene expression (Table I). The simple sequence contingency loci are repeating tracts (microsatellites) in pathogens, such as *Haemophilus influenza* and *Neisseria meningitis*, that are involved in phase variation, a high-frequency gain or loss of the expression of virulence associated with changes in the number of repeats. Slippage of the complementary strands during replication is the accepted mechanism involved in such length changes. The involvement of slipped structures in the genetic instabilities of triplet repeat sequences has probably been studied most extensively. At least 14 hereditary neurological diseases, including myotonic dystrophy, fragile X syndrome, and FRDA, are caused by the massive expansion of CTG·CAG, CGG·CCG, and GAA·TTC, respectively. The capacity of one of the complementary strands in each duplex to preferentially adopt a quasi-stable slipped structure during replication–repair–recombination, thus causing primer realignment, is the mechanism. Also, the first two repeating sequences are flexible and writhed, contributing to their genetic instability.

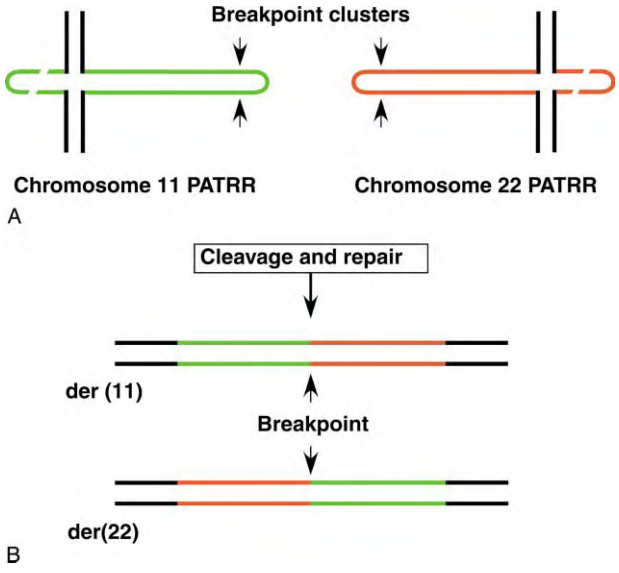



**FIGURE 4** Human t(11;22) translocation. (A) Chromosomes 11 and 22 contain A/T-rich inverted repeats (PATRR) that form cruciform structures cleaved by nucleases. (B) Cleavage sites are repaired by joining chromosome 11 with 22, which yields the translocations der(11) and der(22). Adapted from Kurahashi, H., and Emanuel, B. S. (2001). Long AT-rich palindromes and the constitutional t(11;22) breakpoint. *Hum. Mol. Genet.* **10**, 2605–2617, by permission of Oxford University Press.

## See Also the Following Articles

DNA Supercoiling • Friedreich's Ataxia

## Glossary

**base pairs** Bases associated by hydrogen bonds and hydrophobic forces.

**DNA secondary structure** A DNA conformation (non-*B*-DNA) different from the antiparallel right-handed double helix, *B*-DNA.

**supercoiling** The folding and wrapping of a constrained DNA duplex onto itself.

## Further Reading

Brown, B. A., II, and Rich, A. (2001). The left-handed double helical nucleic acids. *Acta Biochim. Pol.* **48**, 295–312.

Casey, B. P., and Glazer, P. M. (2001). Gene targeting via triple-helix formation. *Prog. Nucleic Acid Res. Mol. Biol.* **67**, 163–192.

Kurahashi, H., and Emanuel, B. S. (2001). Long AT-rich palindromes and the constitutional t(11;22) breakpoint. *Hum. Mol. Genet.* **10**, 2605–2617.

Shafer, R. H., and Smirnov, I. (2001). Biological aspects of DNA/RNA quadruplexes. *Biopolymers* **56**, 209–227.

Siddiqui-Jain, A., Grand, C. L., Bears, D. J., and Hurley, L. H. (2002). Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress a c-*MYC* transcription. *Proc. Natl Acad. Sci. USA* **99**, 11593–11598.

Sinden, R. R. (1994). *DNA Structure and Function.* Academic Press, San Diego, CA.

Soyfer, V. N., and Potaman, V. N. (1996). *Triple-Helical Nucleic Acids.* Springer-Verlag, New York.

Wells, R. D., and Warren, S. T. (1998). *Genetic Instabilities and Hereditary Neurological Diseases*. Academic Press, San Diego, CA.

## Biography

Robert D. Wells is Director of the Center for Genome Research at the Institute of Biosciences and Technology in Houston, TX, and a Professor in the Department of Biochemistry and Biophysics at Texas A&M University. His principal research interest is in alternative DNA structures and their roles in medicine and biology. He holds a Ph.D. from the University of Pittsburgh and received his postdoctoral training at the University of Wisconsin. He has served as the President of the American Society for Biochemistry and Molecular Biology and the Federation of American Societies for Experimental Biology.

Albino Bacolla is a Senior Research Associate at the Institute of Biosciences and Technology in Houston, TX. His research interest is in the biological role of secondary DNA structure and topology. He holds a Ph.D. in Biology from the University of Turin, Italy.

# DNA Sequence Recognition by Proteins

**Arabela A. Grigorescu and John M. Rosenberg**
*University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

**Recognition of specific DNA sequences by proteins refers to the ability of proteins to distinguish and preferentially bind particular DNA sequences in the genome. DNA sequence recognition is vital to cellular processes such as regulation of gene expression, site-specific recombination, DNA replication, and DNA repair; accordingly, in all living organisms, there are many different families of DNA binding proteins. The primary mechanism for this recognition is the formation of a tight complex between the target DNA sequence and a specific protein that has evolved to recognize it. The shapes of the protein and the DNA match each other precisely across the protein–DNA interface. That match is chemically complementary: opposite charges are situated across the interface from each other, as are hydrogen bond donors and acceptors. How they get there is the basis of sequence-specific recognition.**

## What Features of DNA Sequences are Recognized by Proteins?

The Watson-Crick model of the DNA double helix is a uniform structure with a negatively charged phosphate backbone at the exterior and a core of stacked base pairs forming a column up the center. What unique features of a DNA sequence can be recognized by a protein?

### Functional Groups on DNA

The four constituent bases of DNA, adenine (A), thymine (T), guanine (G), and cytosine (C) have unique chemical groups available at their edges to interact with atoms of the protein. These chemical groups – termed functional groups of the DNA bases – are lying on the floors of the two DNA grooves (the concave surfaces of the double helix formed by the edges of the DNA deoxynucleosides (Figure 1)). Because of the asymmetric attachment of the DNA base pairs to the furanose rings, the two grooves of the double-helical DNA molecule have different dimensions. The narrower groove is referred to as the minor groove while the wider groove is referred to as the major groove.

### Complementary Interactions with the Functional Groups

As initially pointed out by A. Rich and co-workers, the A-T and G-C base pairs display unique patterns of hydrogen bond donors and acceptors in the DNA grooves (Figure 1B). Also, the methyl group on the C5 position of the thymine base can interact favorably with $CH_2$ and $CH_3$ groups on amino acid side chains of the protein. The shape of a particular base pair can be recognized by proteins through series of adjacent van der Waals contacts.

Thus, recognition interactions require precise juxtaposition of complementary chemical groups on the protein and on the DNA (chemical complementarity). This complementarity is the basis of recognition, because once a particular protein has achieved the positioning of its functional groups such that they are complementary to those presented by one base sequence, they would not match the functional groups presented by a different sequence.

Water-mediated interactions are also common at protein–DNA interfaces. Polar atoms from amino acid side chains such as Asn, Gln, Thr, and Ser, as well as the amino and carboxyl groups of the protein's main chain, often trap water molecules between the protein and the DNA. The water molecules bridge the protein and the DNA, forming hydrogen bonds to both. Because of the functional groups often involved, these bridging interactions can form simple extensions of the complementarity described above.

### Reading Heads

In order to "read" even a short sequence of DNA, the protein must group appropriate amino acid side chains in close proximity. Indeed, some short sequences are recognized by the single structural element of the protein (sometimes referred to as a reading head) that is brought in close proximity to the functional groups of the DNA bases. These structural elements, often called

 © 2004, Elsevier Inc. All Rights Reserved. 

motifs, provide a scaffold for functional groups that do the reading.

Reading heads can be $\alpha$-helices, $\beta$-sheets, or loops. In some cases, they pre-exist as rigid secondary structural elements in the unbound protein, but in others they represent flexible protein regions that adopt a regular structure in response to DNA binding (many proteins couple local folding with site-specific DNA binding – an idea originally proposed by T. Record based on thermodynamic premises).

In the example shown in Figure 2A, the reading head is an $\alpha$-helix inserted into the DNA major groove. In the example shown, the $\alpha$-helix positions four protein side chains for recognition of a total of three base pairs of the DNA. The dimensions of the protein $\alpha$-helix and of the DNA major groove are ideal for this mode of interaction.

Although not as common as $\alpha$-helices, two stranded $\beta$-sheets have also been observed to serve as reading heads. In the example shown in Figure 2B, the convex side of a curved, two-stranded $\beta$-sheet runs parallel to the surface of the major groove; six amino acid residues (three in each $\beta$-strand) make direct readout interactions with a total of six consecutive DNA base pairs.

### Indirect Readout

Despite the existence of several such simple and attractive examples, in general, direct readout by a rigid fit of $\alpha$-helices and/or $\beta$-sheets to the DNA major groove is not the predominant mechanism employed by proteins for DNA recognition. Only a limited number of sequence-specific DNA-binding proteins exclusively use direct readout, and very often direct readout alone cannot explain the energetic preference of a protein for a particular DNA site. A second, more subtle mechanism for sequence discrimination originates in the distortability of the DNA helix, as shown by structural analysis of a large number of protein–DNA complexes. They reveal that DNA is characterized by remarkable flexibility. In a complex with a protein, the DNA often adopts a conformation that departs significantly from a regular double helix. Several examples given in Figure 3 show that DNA can be, for example, bent, kinked, elongated, and wrapped around a protein. Many lines of experimental evidence suggest that the energetic cost of distorting the DNA into a particular conformation is sequence dependent, and this dependence is exploited by proteins for sequence recognition.

As the DNA is distorted, its chemical groups change their spatial positions. Because the energy required to achieve that distortion is sequence dependent, the resulting spatial positioning of the functional groups reflects the sequence. This is true even for functional groups that do not change with base sequence, such as backbone phosphate groups. Thus, the energy liberated by contacts between a protein and a distorted DNA molecule depend subtly on the distortability of the base sequence. This mechanism of sequence recognition is referred to as indirect readout.

Most site-specific DNA-binding proteins combine the direct and indirect readout mechanisms in order to maximize the information content of DNA sequences. This combination also diversifies the available structural strategies for DNA binding. For example, certain distortions of the DNA helix can alter the dimensions of the DNA grooves such that they can accommodate $\alpha$-helices, $\beta$-sheets, or loops in various orientations. In some cases, dramatic bending of the DNA molecule can completely open up the major or minor groove. In the specific complex of the TATA-binding protein (TBP) and DNA (Figure 3E), the protein is bound on the minor groove side of the DNA, but the DNA is bent by approximately 80° toward the major groove. This distortion alters the minor groove such that it becomes a wide concave surface that is complementary to the curved surface of the TBP protein. In the specific protein–DNA complex of the prokaryotic integration host factor (IHF) (Figure 3F), the DNA molecule is wrapped around the protein. This is possible because the protein induces a bend of approximately 160° in the DNA, and three distinct regions of the DNA molecule are directly contacted and held in place by the IHF protein.

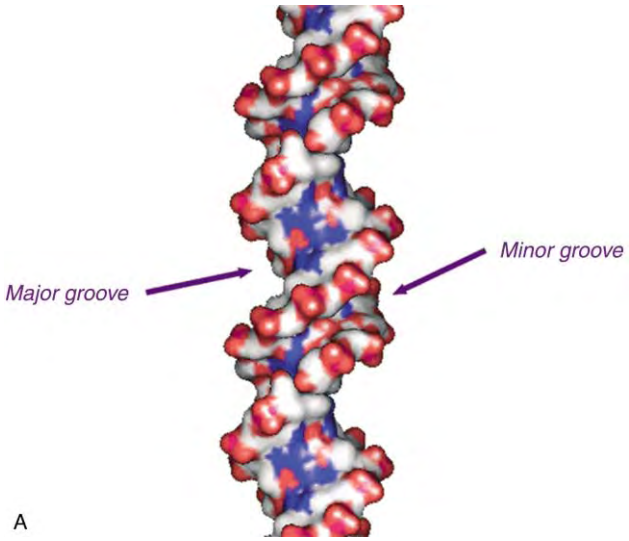
## Structural Motifs in Protein–DNA Recognition

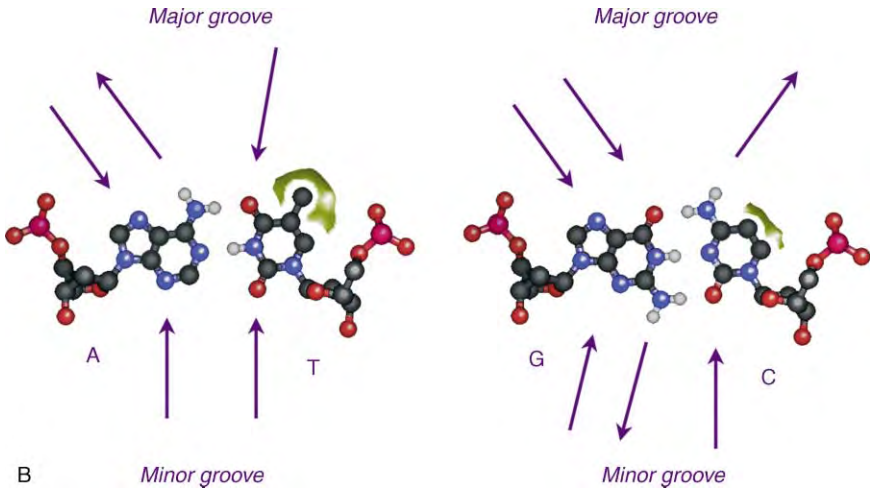
As exemplified in Figure 3, the structural strategies for DNA recognition and binding are remarkably diverse among various classes of DNA-binding proteins. Similar binding strategies and common design principles, however, appear to exist between members of a given protein family; several structural motifs for DNA recognition have been identified in families such as bacterial repressors, eukaryotic transcription factors and other gene regulatory proteins, and particular classes of DNA enzymes.

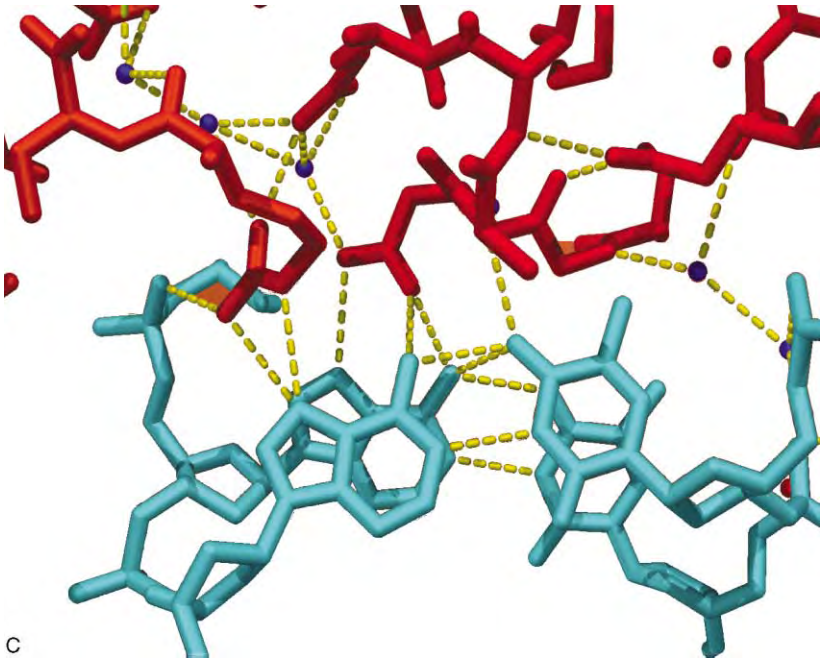
### The Helix-Turn-Helix Motif

Historically, the helix-turn-helix (HTH) motif was one of the first DNA-binding motifs to be identified and analyzed. DNA recognition by the HTH motif has been reviewed by S. Harrison and A. Aggarwall and more recently by C. Garvie and C. Wolberger.

Common to many prokaryotic gene regulatory proteins and also found in eukaryotic homeodomain proteins, the HTH motif consists of two $\alpha$-helices connected by a turn (Figure 3A). One of the $\alpha$-helices (termed the recognition helix) inserts into the major


Major groove
Minor groove
A


Major groove
Major groove
A
T
G
C
B
Minor groove
Minor groove


C

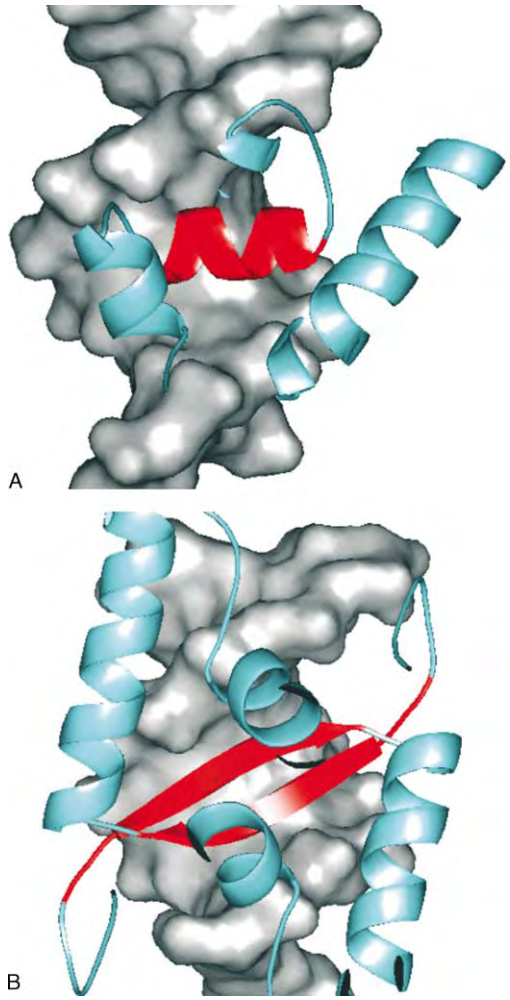



**FIGURE 2** Reading heads in the DNA major groove. The DNA molecular surface is shown in gray. The proteins are shown with a cartoon representation, emphasizing their secondary structure. The amino acid side chains are omitted for clarity. Only regions of the protein that are close to the DNA are visible in the figure. In the examples shown, the DNA reading heads (highlighted in red) are (A) an $\alpha$-helix in $\lambda$ repressor, (B) a two-stranded $\beta$-sheet in MetJ repressor.

groove, making direct or water-mediated contacts with the DNA bases. The other $\alpha$-helix acts primarily to spatially stabilize the motif through hydrophobic interactions in the interior of the two-helix elbow but, in some cases, also contributes to the binding specificity by interacting with the DNA phosphate backbone. Most of the proteins that use this motif for DNA recognition are homodimers; by using two symmetrical HTH motifs, recognition of longer DNA sequences as well as modulated affinity for a series of closely related DNA sequences can be achieved.

Although highly conserved in its internal structure, the HTH motif is highly variable in its precise placement within the major groove of DNA, i.e., the geometry of the DNA–HTH interaction is not as highly conserved. This apparent paradox has been resolved by noting that the HTH motif forms a highly stable bulge on the surface of a protein, a structural requirement for an effective reading head. Thus, although primarily found in DNA-recognition roles, HTH motifs have been found in other situations where there is a structural requirement for a small bulge.

## The Zinc Finger Motif

The zinc finger DNA-binding motif is common to many transcription factors in the human genome. The first zinc finger domains were identified in the structure of *Xenopus* transcription factor IIIA in 1985. This motif consists of a relatively short $\alpha$-helix and a two-stranded antiparallel $\beta$-sheet (Figure 3C). The fold is stabilized by a core of residues (most commonly two cysteines and two hystidines) coordinating a $Zn^{2+}$ ion. Typically, the N-terminal part of the $\alpha$-helix is inserted in the major groove, contacting 3 to 4 DNA bases. Many gene regulatory proteins in eukaryotes contain several copies of this motif connected by linkers; variations in the number and sequence of the zinc finger motifs and the length and flexibility of the linker regions allow transcription factors and other similar proteins to recognize long and complex DNA target sites using a relatively simple structural motif. Here, too, the zinc finger forms a bulge found most often, but not exclusively, as a DNA recognition element.

## The Leucine Zipper Motif

The basic leucine zipper (bZIP) binding motif is another structural motif employed by eukaryotic gene regulatory proteins for DNA recognition. It consists of two long, approximately parallel $\alpha$-helices wrapped partially around each other to form a type of structure called a coiled coil. The contacts between the $\alpha$-helices are stabilized by hydrophobic interactions made by a series of leucine residues. The N termini of the $\alpha$-helices are the DNA reading heads that insert into the major groove (Figure 3B). They are unstructured in the absence of DNA; thus, the basic leucine zipper motif couples local

**FIGURE 1** (A) The molecular surface of B-DNA; the major groove and minor groove are indicated. (B) The A-T and G-C DNA base pairs. The coloring scheme for the atoms is carbon, black; oxygen, red; nitrogen, blue; hydrogen, white. The arrows illustrate the hydrogen-bonding potential of the functional groups and point from the hydrogen bond donor toward the hydrogen bond acceptor. The methyl group of the thymine and C-H groups of cytosine can be contacted by the protein through van der Waals interactions. (C) View of a typical protein–DNA recognition interface. The hydrogen bonds between the protein and the DNA are indicated by dashed lines.

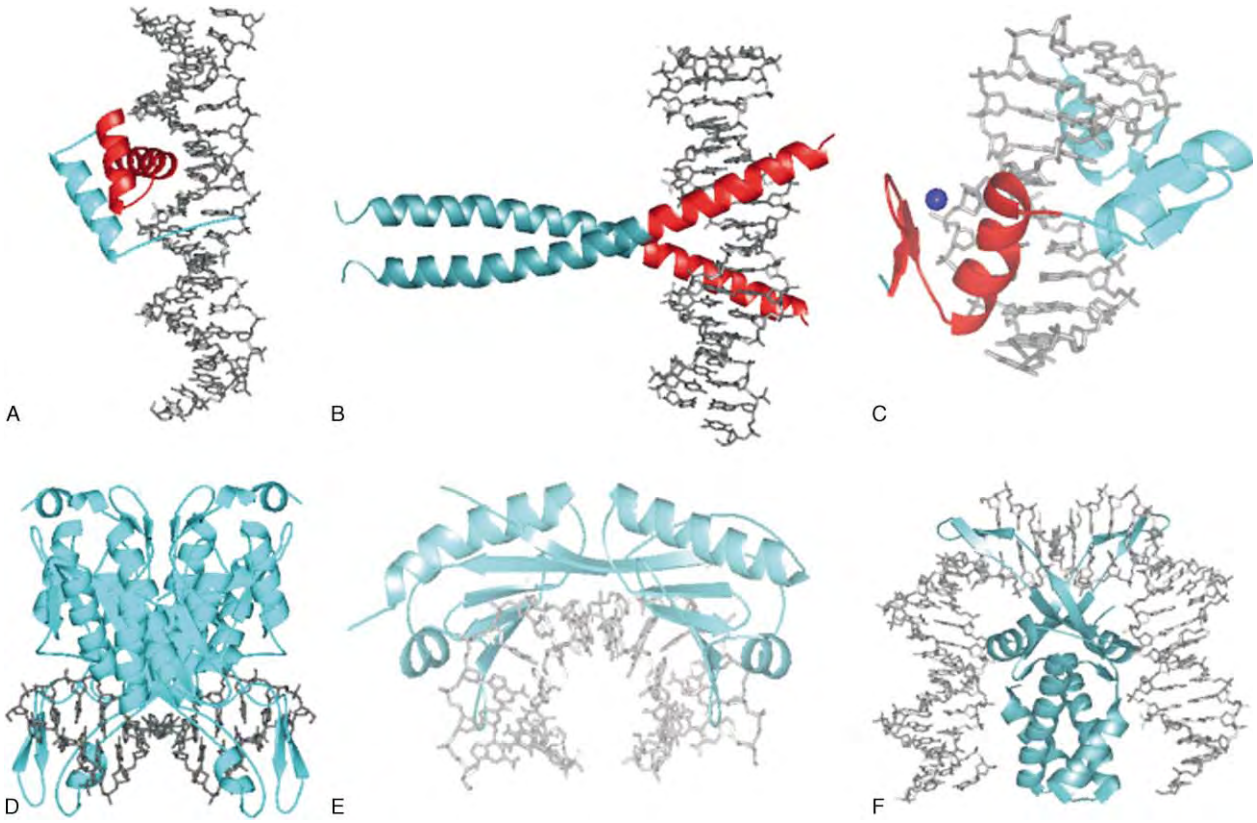



**FIGURE 3** Diverse structural strategies for DNA binding and recognition are employed by proteins. Top: examples of structural motifs (highlighted in red) for DNA recognition: (A) helix-turn-helix (HTH) in prokaryotic λ repressor, (B) leucine zipper (bZIP) in yeast transcriptional activator GCN4, (C) zinc finger in mouse transcription factor ZIF268. Bottom: examples of protein–DNA complexes with significant DNA distortion: (D) bacterial type II endonuclease *Eco*RI, (E) yeast TATA-binding protein (TBP), (F) prokaryotic integration host factor (IHF).

folding with DNA recognition. T. Hakoshima and co-workers have reviewed the structural basis of DNA recognition by bZIP transcription factors.

## Diverse Structural Strategies are Employed by Proteins for DNA Recognition

In general, structural DNA-binding motifs such as helix-turn-helix, zinc fingers, and leucine zipper are used by proteins as DNA-binding modules. In some proteins, they facilitate multiple independent intermolecular contacts and allow modulated affinity for a series of related sites. Proteins with stringent sequence specificity, such as restriction enzymes, use a more integrated recognition mode. Several structural elements of the protein are involved in DNA recognition over a large structural area, but these interactions are not independent. Extensive networks of hydrogen bonds and other favorable interactions form cooperatively at the molecular interface upon site-specific binding; such networks contribute to the specificity and stability of these complexes. These networks often extend to enzymatic active sites, e.g., the cleavage sites of restriction enzymes, thereby integrating recognition and cleavage.

One of the few common trends among the various families of DNA-binding proteins is that most proteins bind to DNA as homodimers, heterodimers, or pseudo-dimers. This strategy has several purposes: first, it is a relatively simple way to bring two or more DNA reading heads in proximity at the interface and to create a larger interface from a globular protein and a helical DNA molecule. Second, additional specificity can be achieved by coupling DNA recognition interactions with quaternary structure interactions within the dimer. Third, the symmetry or the asymmetry of these dimers is often required for the specific function of the protein. For example, type II bacterial restriction endonucleases that recognize short palindromic DNA sites make double-strand breaks in DNA by positioning the two scissile bonds in the two symmetric catalytic sites of the homodimeric enzyme. Proteins that are required to bind DNA in a precise orientation are often

pseudodimers or heterodimers; such examples include eukaryotic TATA-binding protein and prokaryotic integration host factors, respectively.

## Conclusion

As the number of structurally characterized protein–DNA complexes continues to expand, it is becoming evident that evolution has engineered a diversity of DNA-binding proteins and DNA recognition modes. By correlating the structural analysis with energetic and functional characterization of DNA-binding proteins, it is becoming increasingly clear that DNA recognition is tightly coupled with the protein function. Conservation of DNA-binding motifs and design principles within protein families reflects the fact that the structural strategies and energetic principles for DNA recognition have been shaped by evolution such that proteins can achieve simultaneously both the desired level of sequence specificity and optimal functionality in their complexes with DNA.

### See Also the Following Articles

DNA Base Excision Repair • DNA Replication: Eukaryotic Origins and the Origin Recognition Complex • DNA Replication: Initiation in Bacteria • DNA Replication, Mitochondrial • Recombination-Dependent DNA Replication • Transcription-Coupled DNA Repair, Overview • Zinc Fingers

### Glossary

**direct readout** Recognition of the identity of the functional groups of the DNA bases by a protein through specific interactions (hydrogen bonds, hydrophobic, van der Waals contacts, etc).

**DNA distortion in a protein–DNA complex** A significant departure of the DNA conformation within the complex from that of the free DNA (with the same sequence) in solution (under physiological conditions).

**DNA reading head** A structural element or motif of the protein that makes specific contacts with functional groups of the DNA bases when it is brought in close proximity to those bases; typically contains several amino acid residues that are involved in the recognition interaction.

**indirect readout** Recognition of the sequence-dependent conformational features of the DNA, including the energetic cost of distorting the DNA into a particular conformation.

**sequence specificity in DNA binding** The preference of a given protein for binding DNA of a particular sequence over binding DNA independently of its sequence (nonspecific binding); typically expressed via the differential free energy of binding.

### Further Reading

Fujii, Y., Shimizu, T., Toda, T., Yanagida, M., and Hakoshima, T. (2000). Structural basis for the diversity of DNA recognition by bZIP transcription factors. *Nat. Struct. Biol.* **7**, 889–893.

Garvie, C. W., and Wolberger, C. (2001). Recognition of specific DNA sequences. *Mol. Cell* **8**, 937–946.

Harrison, S. C., and Aggarwal, A. K. (1990). DNA recognition by proteins with the helix-turn helix motif. *Annu. Rev. Biochem.* **59**, 933–969.

Miller, J., McLachlan, A. D., and Klug, A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO J.* **4**, 1609–1614.

Pabo, C. O., and Nekludova, L. (2000). Geometric analysis and comparison of protein–DNA interfaces: Why is there no simple code for recognition? *J. Mol. Biol.* **301**, 597–624.

Rosenberg, J. M. (1991). Structure and function of restriction endonucleases. *Curr. Opin. Sruct. Biol.* **1**, 104–113.

Seeman, N. A., Rosenberg, J. M., and Rich, A. (1976). Sequence specific recognition of double helical nucleic acids by proteins. *Proc. Natl. Acad. Sci. USA* **73**, 804–808.

Steitz, T. A. (1990). Structural studies of protein-nucleic acid interaction: The sources of sequence-specific binding. *Quart. Rev. Biophys.* **23**, 205–280.

Tateno, M., Yamasaki, K., Amano, N., Kakinuma, J., Koike, H., Allen, M. D., and Suzuki, M. (1997). DNA recognition by beta-sheets. *Biopolymers* **44**, 335–359.

Wodak, S. J., and Janin, J. (2002). Structural basis of macromolecular recognition. *Adv. Prot. Chem.* **61**, 9–73.

Wolberger, C. (1993). Transcription factor structure and DNA binding. *Curr. Opin. Struct. Biol.* **3**, 3–10.

### Biography

John M. Rosenberg is a member of the Department of Biological Science at the University of Pittsburgh. He holds a Ph.D. from the Massachusetts Institute of Technology. His major research interest is in the structural basis of sequence-specific DNA–protein interactions.

Arabela A. Grigorescu is a research associate in the Department of Biological Sciences at the University of Pittsburgh. She holds a Ph.D. from the University of Pittsburgh.

# DNA Supercoiling

**Tao-shih Hsieh**
*Duke University Medical Center, Durham, North Carolina, USA*

**DNA supercoiling describes a higher-order DNA structure. The double-helical structure of DNA entails the interwinding of two complementary strands around one another and around a common helical axis. The writhing of this helical axis in space defines the DNA superhelical structure (DNA tertiary structure). For a circular DNA or a linear DNA with its ends anchored to create a loop, there is a tight topological coupling between the DNA superhelical structure and the double-helical structure (DNA secondary structure). Hence, DNA superhelicity can influence the DNA winding/unwinding, thereby affecting the biological functions of DNA. In nature, there exists a ubiquitous class of enzymes, DNA topoisomerases, that can mediate the topological transformation in DNA molecules.**

## Background

The beauty and elegance of the double-helical structure of DNA, as first proposed by Watson and Crick in 1953, also imparts critical functions of DNA. However, the unwinding of the DNA double helix during the process of replication, transcription, and recombination creates intriguing topological problems. The potential topological problem intrinsic to a circular, double-stranded DNA molecule was first noted by Cairns in 1963. In his autoradiographic analysis of chromosome replication in the bacterium *Escherichia coli*, Cairns pointed out that the bihelical DNA structure of a circular molecule poses a topological constraint on the strand separation necessary in the process of DNA replication. Vinograd and his associates about the same time demonstrated that an animal virus, polyomavirus, also has a circular genome. Furthermore, the helical axis itself winds and turns in space, thus producing a superhelix. Since then, circular genomes have been discovered in many organisms, including most bacteria, bacterial plasmids, and organelles such as mitochondria and chloroplasts. For example, Figure 1 shows an electron micrograph of a small bacterial plasmid DNA. The DNA superhelicity results in a duplex making crossovers (intersections) with itself. For most circular DNAs isolated from natural sources, the number of superhelical turns is directly proportional to the length of DNA. There are approximately six to eight superhelical turns per 1 kb DNA. We discuss the quantitative details in later sections.

The biological significance of DNA superhelicity was first realized when Vinograd and his co-workers discovered that there is a tight coupling between the DNA secondary structure and superhelical structure. Therefore, DNA superhelicity can influence the unwinding and rewinding of DNA duplex, and vice versa. Because such a coupling is direct, it allows one a rare opportunity in molecular biology to design experiments to gain insight into the structure of DNA and to probe the mechanism of the enzymes that affect DNA structure. The theoretical consideration of the coupling between DNA secondary and tertiary structures, based on mathematical topology, was later extended in the treatises by Fuller, White, and Crick. It should be pointed out that such a topological coupling also applies to linear DNA with anchored sites to create the loop structure that is found in the chromosomes of essentially all cells.

Nature has evolved unique enzymes to solve the topological problems that arise during the process of unwinding/rewinding DNA helix. The first such enzyme, topoisomerase I from the bacterium *E. coli*, discovered by Wang in 1970, can remove negative supercoils efficiently. Many new families of topoisomerases have since been discovered, and these enzymes are present in all organisms and have essential biological functions.

## Quantitative Relationship between Twist and Supercoil

For a circular DNA without any nicks, in which both DNA strands are covalently continuous, the sum of DNA twists (*Tw*) and writhes of the DNA helical axis (*Wr*), a measure of DNA superhelical structure, is invariant. Because the algebraic sum of these two numbers is equal to the number of times that one of the DNA strands winds around its complementary strand in space, it defines the topological linkage between DNA strands, termed the linking number (*Lk*).

 © 2004, Elsevier Inc. All Rights Reserved. 



**FIGURE 1** An electron micrograph of a bacterial plasmid DNA. The DNA has about 3 superhelical turns, as evidenced by the number of crossovers made by the DNA duplex.

We therefore have the following equation:

$$Tw + Wr = Lk$$

where $Lk$ is a constant for a covalently closed circular DNA. $Tw$ is related to the DNA secondary structure, the bihelical winding of two DNA strands, and it can be calculated if one knows the size of the DNA and the average helical pitch in this DNA. For example, in a 1-kb B-form DNA with a helical pitch of 10.5 bp/turn, $Tw = 95$. $Wr$ is a measure of the superhelical structure of DNA. It relates to the number of times that the helical axis crosses itself in space. The higher the $Wr$, the more supertwisted the DNA. $Lk$ is a topological quantity and defines the total linkage between the two complementary strands. Whereas $Tw$ and $Wr$ can be any real number, $Lk$ is always an integer. This is because, for a closed circular DNA, strands have to wind around one another an integral number of turns to avoid nicks or gaps.

## Handedness and the Sign of *Tw* and *Wr*

An important feature for all three parameters is that each has a sign, positive or negative, depending on the handedness of the double helix and superhelix. Because most of the DNA duplexes are right-handed, we define right-handed as positive and left-handed as negative. We can define whether the crossover of two curves in space is left-handed or right-handed if an axis is clearly marked. For example, Figure 2 shows two curves, denoted as two arrows in space, one making a left-handed cross and the other a right-handed one. The axis is shown as a thin vertical line going through the crossover. The significance of the definition of the axis can be visualized in this figure as well. If one chose instead a horizontal line as the axis, then the assignment of handedness is reversed. Figure 2B would then be left-handed and Figure 2A would be right-handed. For the duplex DNA structure, the axis definition is rather intuitive and straightforward. The helical axis is the axis around which the two strands are wrapped with respect to one another. For the DNA superhelix, it is necessary to define an axis in a manner that is consistent with what we defined for secondary structure. An example is shown in Figure 3. A figure-eight superhelix is the simplest possible DNA supercoil. If one lays down the figure-eight superhelix horizontally, then the axis is the vertical line going through the crossover. With this definition of axis, the superhelix shown in Figure 3A has a left-handed crossover, and thus it has a negative superhelical turn ($Wr = -1$). Figure 3B has a positive superhelical turn ($Wr = +1$).

Because it is not practical to measure the absolute value of $Wr$ or $Lk$, they are usually determined experimentally with respect to a reference state, DNA in a relaxed state. The linking number and writhe for the relaxed state are denoted with a subscript zero.

Because $Wr_0 = 0$, the measure of DNA supercoils, $Wr$, is equal to the writhe difference with respect to the reference state:

$$Wr = \Delta Wr = Wr - Wr_0$$

Assuming that $Tw$ for the supercoiled and relaxed DNA is about the same ($Tw = Tw_0$), then it follows that:

$$\Delta Wr = \Delta Lk$$

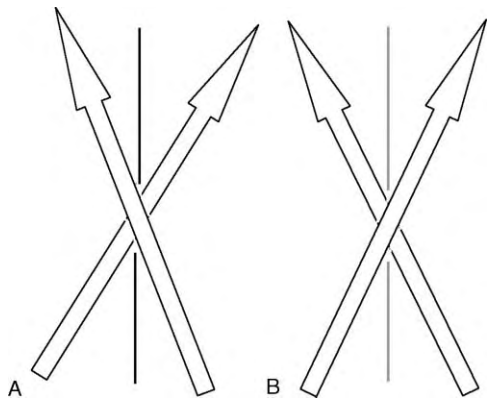



**FIGURE 2** Diagrams of (A) a left-handed crossover and (b) a right-handed one. The arrows shown here simply serve to illustrate the point that the two perpendicular axes, one vertical and one horizontal, are not equivalent. The vertical line has been chosen arbitrarily as the axis for determining handedness.

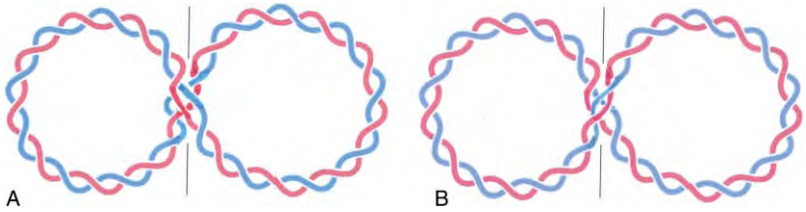



**FIGURE 3** Diagram of a DNA with (A) a left-handed supercoil and (B) with a right-handed supercoil. Notice the two complementary strands (in red and blue) are wound in a right-handed helix. The axis for determining the handedness of the superhelical turn is shown as a vertical line going through the DNA crossover.

Because *Lk* is a topological invariant, it does not change for a given DNA in different environments. It is thus a more useful parameter for tracking the structure of DNA. $\Delta Lk$ is thus frequently used to mark the superhelicity of DNA. If $\Delta Lk$ is negative, DNA is underwound; the absolute value of ΔLk in this case gives a measure of negative supercoils or linking deficiency. If $\Delta Lk$ is positive, then the DNA is overwound; its absolute value relates to the number of positive superhelical turns. DNA superhelicity can also be expressed as a fraction with respect to the linking number in a relaxed DNA. This is usually referred to as superhelical density, $\sigma$.

$$\sigma = \Delta Lk/Lk_0 = \Delta Lk/Tw_0$$

because $Lk_0 = Tw_0$, when $Wr_0 = 0$.

Most of the circular DNAs isolated from natural sources are negatively supercoiled. An interesting exception is the DNA in hyperthermic bacteria, which is positively supercoiled. For the numerous negatively supercoiled DNAs studied so far, the superhelicity mostly falls within a narrow range, with $\sigma$ between $-0.06$ and $-0.08$.

## Biological Effects of Supercoiling and Enzymes That Can Change DNA Supercoiling

To access the genetic information embedded within the double helix, most of the biological processes associated with critical functions of DNA involve unwinding or rewinding of the twin DNA strands. With a covalently closed circular DNA for which *Lk* stays constant, any change in *Tw* will be compensated by a change in *Wr* that is equal in magnitude but opposite in sign:

$$\Delta Tw = -\Delta Wr$$

This quantitative coupling between the DNA secondary structure and superhelical structure suggests that supercoiling can profoundly influence DNA structure and function. DNA with supercoils, either positive or negative, is thermodynamically less stable than its relaxed counterpart. For a negatively supercoiled DNA, any reactions associated with a reduction in the negative superhelical turns ($\Delta Wr > 0$) are energetically favored. These reactions will also be coupled with the unwinding of the DNA duplex because $\Delta Tw < 0$ if $\Delta Wr > 0$. Therefore negative DNA supercoiling facilitates the unwinding of DNA. In many instances, it has been demonstrated that replication, transcription, and recombination, all of which require the unwinding of DNA, are promoted by negative supercoiling. In an interesting corollary, DNA with positive supercoils will favor winding and disfavor unwinding of duplex structure (i.e., $\Delta Tw > 0$ if $\Delta Wr < 0$). It has been hypothesized that positive supercoiling found in some of the hyperthermophiles serves to stabilize the DNA double-helical structure in the extreme temperatures at which these organisms find home.

Nature has harnessed the use of DNA supercoiling for regulating the biological functions of DNA; it has also developed tools to modulate supercoiling. A ubiquitous class of enzymes, DNA topoisomerases, evolved to carry out the topological transformation in DNA. In order to break the hold of the topological constraint on closed circular DNA, these enzymes can make transient and reversible DNA breaks. There are two important features in these topoisomerase-mediated breaks in comparison with those made by nucleases. One is that a specific tyrosyl residue in the enzyme is joined covalently to the phosphate at the breakage site through a phosphodiester bond. Because this enzyme–DNA bridging bond is made at the expense of the neighboring DNA backbone bond, there is no significant energy change associated with the process of strand scission, thus assuring that the cleavage/religation is readily reversible. The enzyme-mediated DNA breakage is also accompanied by strand passage through the break or a rotation of the strand with the transient break around the intact strand. Both processes result in a change of DNA supercoiling or other topological transformation in DNA. Indeed, genetic experiments have demonstrated essential functions of these enzymes in the cell. These results also

underscore the importance of supercoiling in the biological functions of DNA.

## See Also the Following Articles

DNA Secondary Structure • DNA Topoisomerases: Type I • DNA Topoisomerases: Type II

## Glossary

**DNA twist** The number of times that two complementary strands wind around one another in the double-helical structure.

**kilobase (kb)** One thousand base pairs.

**linking number** The number of times that DNA strands wind around one another in space. For a closed circular DNA, it is an integer and a topological invariant.

**supercoil** The winding of the DNA helical axis in space.

**topoisomerase** An enzyme that can change supercoiling or other aspects in the topological structure without altering any covalent structure in DNA.

## Further Reading

Cozzarelli, N. R., and Wang, J. C. (eds.) (1990). *DNA Topology and Its Biological Effects*. Cold Spring Harbor Laboratory Press, New York.

Wang, J. C. (1980). Superhelical DNA. *Trends Biochem. Sci.* **5**, 219–221.

Wang, J. C. (1994). Appendix I: An introduction to DNA supercoiling and DNA topoisomerase-catalyzed linking number changes of supercoiled DNA. In *Advances in Pharmacology Vol. 29B, DNA Topoisomerases: Biochemistry and Molecular Biology* (L. Liu, ed.) pp. 257–270. Academic Press, San Diego, CA.

## Biography

Tao-shih Hsieh is a Professor in the Department of Biochemistry at Duke University Medical Center, Durham, NC. His general research area is in the structure and function of eukaryotic chromosomes. He received a Ph.D. from the University of California at Berkeley and did his postdoctoral fellowship at the Stanford University Medical Center. His research has contributed to the discovery of new DNA topoisomerases and the unraveling of their biological functions.

# DNA Topoisomerases: Type I

**James J. Champoux**
*University of Washington, Seattle, Washington, USA*

**The large size of DNA molecules and the double-helical nature of DNA create unique topological problems during replication, transcription, recombination, and chromatin remodeling that are solved by a family of enzymes called DNA topoisomerases. Members of the type II subfamily of DNA topoisomerases alter the supercoiling of DNA and disentangle chromosomes by introducing temporary double-strand breaks into the DNA. Type I DNA topoisomerases, the subject of this review, manage DNA topology in the cell by transiently cleaving only one of the two DNA strands.**

## Reactions Catalyzed by Type I DNA Topoisomerases

To introduce a temporary single-strand break into duplex DNA, type I DNA topoisomerases must catalyze the cleavage and subsequent religation of a DNA strand. Since these two reactions occur without an external energy source such as ATP, cleavage cannot result from simple hydrolysis of a phosphodiester bond in the DNA. Instead, a covalent enzyme–DNA intermediate is generated that makes the religation step energetically feasible. The formation of the covalent intermediate involves nucleophilic attack by the O-4 atom of the active site tyrosine in the enzyme on a phosphodiester bond in the DNA to produce a phosphodiester bond between the tyrosine and the DNA and leave a free DNA hydroxyl end. DNA religation and release of the enzyme is the reverse reaction with the oxygen of the free DNA hydroxyl acting as the nucleophile. The type I enzymes display a loose preference for certain nucleotides in the vicinity of a cleavage site, and therefore a cleavage site typically occurs every 5–20 base pairs along the DNA.

Type I topoisomerases act on closed circular DNAs to change the number of times one strand winds around the other, a parameter referred to as the linking number of the DNA. Changes in the linking number are reflected in a reduction or an increase in the supercoiling of a plasmid DNA, a property that is most often measured by gel electrophoresis. In addition to altering the supercoiling of a plasmid DNA, many type I topoisomerases are capable of catalyzing a number of other transactions involving both single- and double-stranded DNAs. Most of these enzymes can catenate (interlock), decatenate, knot, and unknot single-stranded DNA circles. The same series of reactions can be carried out with duplex circular DNAs providing at least one of the circular molecules possesses a nick or gap. In some cases, the enzymes can facilitate the interwinding required for the renaturation of two complementary single-stranded circular DNAs, a reaction that could be important during homologous recombination. Interestingly, topoisomerase V, which has only been described in the hyperthermophilic archaeon *Methanopyrus kandleri*, possesses, in addition to the usual topoisomerase activity, an apurinic/apyrimidinic site-processing activity that would appear to implicate the enzyme in DNA repair. Finally, with certain unusual DNA substrates, a block to religation leads to permanent suicide cleavage and the enzyme remains covalently linked to the DNA.

## Classification, Nomenclature and General Properties

Type I topoisomerases are classified into two structurally and mechanistically distinct subfamilies based on which DNA end becomes covalently attached to the enzyme during the cleavage reaction: type IA enzymes attach via a tyrosine phosphodiester linkage to the 5′ end of the DNA, whereas type IB enzymes attach to the 3′ end of the DNA. Table I lists the known type I DNA topoisomerases in the two subfamilies with their common names and origins. The common names have generally been assigned in the order of discovery using odd Roman numerals (even Roman numerals are similarly used for type II DNA topoisomerases). Type I enzymes with unusual properties or origins have been given unique names (reverse gyrase, poxviral topoisomerase, and mitochondrial topoisomerase). The recently described IB enzymes in some eubacteria are currently referred to as bacterial topoisomerases IB.

The three categories of type IA enzymes listed in Table I can be distinguished on the basis of the types of

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Type I DNA Topoisomerases**

| Subfamily | Common name | Source | Structure |
|---|---|---|---|
| IA | Topoisomerase I | All eubacteria and some archaebacteria | Monomer |
| IA | Topoisomerase III | Some eubacteria and most eukaryotes | Monomer |
| IA | Reverse gyrase | All hyperthermophilic eubacteria and archaea | Monomer |
| IA | Reverse gyrase | Archaeon *Methanopyrus kandleri* | Heterodimer |
| IB | Topoisomerase I | Nucleus of all eukaryotes | Monomer |
| IB | Mitochondrial topoisomerase | Mitochondria of higher eukaryotes | Monomer |
| IB | Poxviral topoisomerase | All members of poxviridae family | Monomer |
| IB | Topoisomerase V | Archaeon *Methanopyrus kandleri* | Monomer |
| IB | Topoisomerase IB | Some eubacteria (see Table II) | Monomer |
| IB | Topoisomerase I | Trypanosomatids *Trypanosoma brucei* and *Leishmania donovani* | Heterodimer |

reactions they catalyze. Topoisomerases I relax negative but not positive supercoils in plasmid DNAs; but since relaxation does not go to completion, some residual negative supercoils remain in the product. Topoisomerases III require hypernegatively supercoiled plasmid DNA as a substrate and again relaxation is incomplete. Interestingly, topoisomerases III are much more proficient than the topoisomerases I in DNA catenation and decatenation (see below). Reverse gyrases, which are only found in hyperthermophilic eubacteria and archaebacteria, introduce positive supercoils into plasmid DNAs at the expense of ATP hydrolysis. All of the type IA enzymes require $Mg^{2+}$ and are monomeric with the exception of the reverse gyrase from the archaeon *Methanopyrus kandleri* which is a heterodimer.

The type IB DNA topoisomerases are capable of relaxing both positive and negative supercoils in a reaction that does not require ATP or divalent cations. The reactions go to completion to produce a completely relaxed set of plasmid DNA topoisomers. With the exception of the recently discovered heterodimeric topoisomerases I from trypanosomatids, all of the type IB enzymes are monomeric.

## Type IA DNA Topoisomerases

### Protein Domains

All type IA topoisomerases (Table I) share a highly conserved "cleavage/strand passage" domain that contains the active site tyrosine. This domain is also responsible for promoting the structural change in the DNA during the interval between the cleavage and religation reactions that results in a linking number change (see below) (Figure 1, red boxes). As indicated in Figure 1, all type IA enzymes contain a poorly conserved

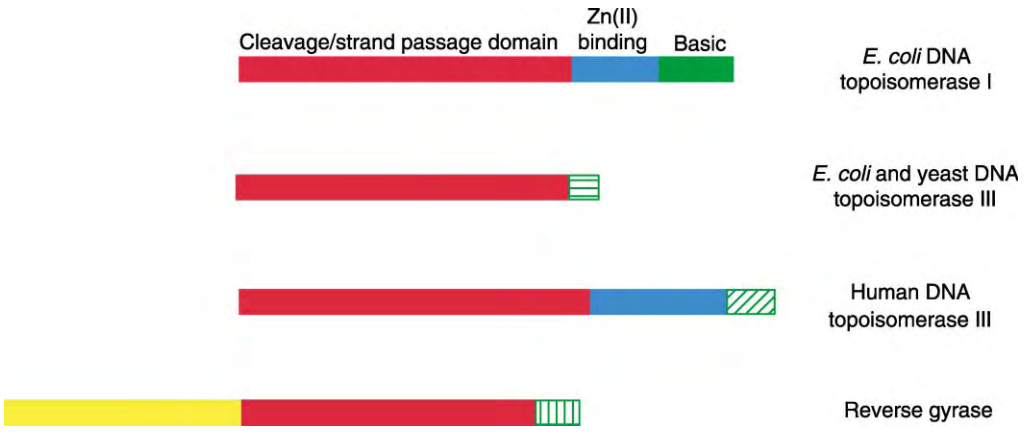



**FIGURE 1** Domain structure and sequence relationships between type IA DNA topoisomerases. The domain structure of representative type IA topoisomerases is denoted by colored boxes. The names of the domains for the prototypic *E. coli* DNA topoisomerase I are given along the top. The cleavage/strand passage domains shared by all the IA enzymes are shown in red. Green is used to denote the basic C-terminal domains, but the different types of fill for these boxes indicate that these domains are poorly conserved. Some type IA enzymes contain a Zn(II) binding domain shown in blue. The helicase-like domain of reverse gyrase is shown in yellow.

basic C-terminal domain (solid or hatched green boxes) and some contain a Zn(II) binding domain as well (blue boxes). These latter two features appear to be important for the interaction of the enzyme with DNA. Finally, reverse gyrases contain an N-terminal domain, which resembles the ATPase domains of helicases (yellow box), and is connected to two domains that are structurally very similar to the cleavage/strand passage and basic domains of the typical type IA topoisomerases.

## Crystal Structure of the Conserved Cleavage/Strand Passage Domain

The crystal structure of the cleavage/strand passage domain of *E. coli* DNA topoisomerase I shown in Figure 2 provides key insights concerning the substrate preference of the enzyme and the mechanism of DNA relaxation. Notably, the cleavage/strand passage domains of all type IA topoisomerases bear a strong resemblance to the *E. coli* structure. The hallmark of the crystal structure is a toroidal shape in which the diameter of the hole in the center of the torus is sufficient to accommodate either single- or double-stranded DNA.

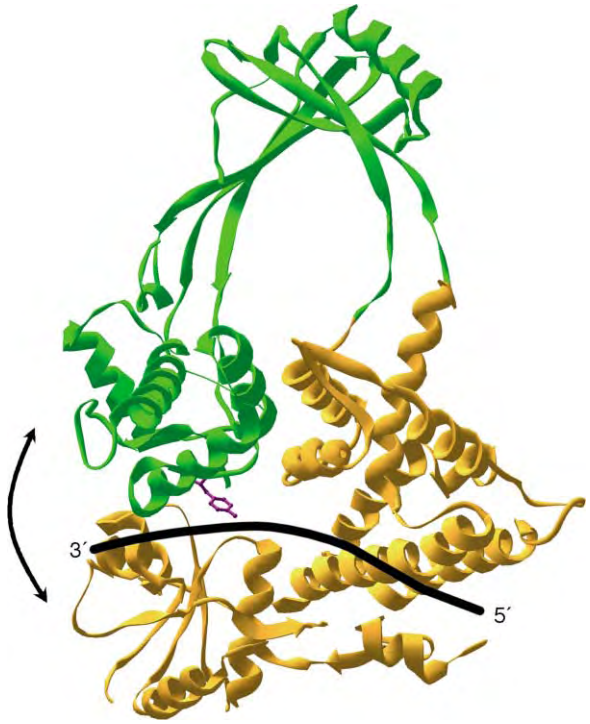



**FIGURE 2** A ribbon diagram showing the crystal structure of the cleavage/strand passage domain of *E. coli* DNA topoisomerase I (pdb entry 1ecl) drawn with Swiss-Pdb Viewer software (Glaxo Wellcome Experimental Research). The approximate path of the bound single-stranded substrate DNA is indicated by the solid black line, and the active site tyrosine is shown in magenta. The two regions of the protein that move relative to each other (double-headed arrow) to open and close the torus during the strand passage reaction and to release the DNA are shown in green and orange.

The requirement for a negatively supercoiled substrate and the inability to completely relax negative supercoils is best explained by supposing that these enzymes will only bind an otherwise duplex DNA substrate if it contains a single-stranded region resulting from local unwinding of the helix. A plasmid DNA that is highly negatively supercoiled is energetically disposed toward helix unwinding, which explains why such a DNA is a good substrate for the enzyme. However, relaxation ceases when the negative supercoiling falls to a level below which there is insufficient energy to promote the required opening of the helix. As shown in Figure 2, a single strand of DNA (solid black line) binds to a narrow groove on the cleavage/strand passage domain of the enzyme in close proximity to the active site tyrosine (magenta).

## Enzyme-Bridging Mechanism for Strand Passage

To change the linking number of a closed circular DNA during relaxation, one strand of DNA must pass through a break in the other strand. Knotting, catenation, and decatenation of either single or double-stranded DNAs similarly require such a strand passage event. The strand passage reaction for type IA topoisomerases occurs by what is referred to as an enzyme-bridging mechanism. Once the scissile DNA strand is cleaved, both DNA ends remain tightly associated with the enzyme; the 5′ end is bound covalently to the active site tyrosine and the 3′ end is bound noncovalently to the enzyme. To orchestrate strand passage, the enzyme undergoes a conformational change in which the top half of the protein containing the 5′ end of the cleaved strand (Figure 2, shown in green) lifts upward to generate a gate in the DNA through which another strand of DNA is passed. After strand passage, the broken strand is religated and the enzyme opens up a second time to release the strand that had been passed into the hole of the torus. A correlate of this model is that the linking number can only be changed in steps of one and this prediction has been verified biochemically.

This same scheme can explain how the enzyme can catenate or decatenate a DNA containing a nick or gap. However, it is unclear whether the DNA that passes through the temporary gate in the cleaved strand is captured in the hole of the torus before strand cleavage and is then passed out of the hole after cleavage or vice versa as described above.

## Reverse Gyrase Mechanism

Despite the presence of a helicase-like ATPase domain in the N-terminal region of reverse gyrases, these enzymes

lack helicase activity when assayed under conditions that would require processive translocation along the DNA. Instead, the binding of the N-terminal domain to DNA is believed to simply unwind a region of the helix. Subsequently, in a reaction dependent on ATP, one of the two strands is cleaved and the other strand is passed through the resultant gate by the cleavage/strand passage domain present in the C-terminal half of the molecule. The key to positive supercoiling is that the strand passage event that occurs in the presence of ATP is directional such that the linking number of the DNA is increased and therefore the DNA ends up positively supercoiled. The structural basis for the unidirectional nature of the strand passage event remains unknown.

## Type IB DNA Topoisomerases

### Domain Structure and Sequence Conservation

Type IB DNA topoisomerases are present in the nucleus of all eukaryotic cells and the mitochondria of higher eukaryotes, as well as in at least one archaeon and some eubacteria (Table I). The typical eukaryotic type IB enzyme possesses the four domains shown in Figure 3. A highly charged and poorly conserved N-terminal domain (red box) is followed by the core domain (blue box), which binds DNA and contains most of the catalytic residues. The active site tyrosine is found in the C-terminal domain (yellow box) that is connected to the remainder of the protein by a poorly conserved linker region (orange box).

All of the other type IB enzymes share at least partial sequence and structural homology with the catalytically important core domain as can be seen from the color scheme in Figure 3 (blue boxes). Where present, the sequence of the mitochondrial enzyme is very similar to the nuclear enzyme with the exception of the N-terminal region (green box), which contains the organelle targeting signals. The eubacterial IB enzyme and the vaccinia topoisomerase are very similar to each other, but they lack most of the core domain as well as the conserved C-terminal domain that is characteristic of the other IB enzymes; instead, they share unique N-terminal and C-terminal regions (white and magenta boxes). The topoisomerase I found in the trypanosomatids is a heterodimer with one subunit containing the catalytic core (blue box) and the other subunit containing a region homologous to the C-terminal domain of the prototypic eukaryotic sequence (yellow box).

### Crystal Structure of Human Topoisomerase I

Two views of the crystal structure of human topoisomerase I (missing the N-terminal domain) with a bound 22 base pair duplex oligonucleotide are shown in Figure 4. The protein is a bi-lobed structure that clamps completely around the DNA with the active site tyrosine (shown in black in Figure 4A) juxtaposed to the scissile phosphate. The linker region comprises the coiled-coil structure that protrudes conspicuously from the bottom portion of the enzyme and has an unknown function (Figure 4A). To release the DNA, the top half of the protein (shown in blue) must shift upward relative to the bottom half as indicated in Figure 4B by the double-headed arrow. Likewise, DNA binding requires that the protein clamp be in an open conformation. The region of

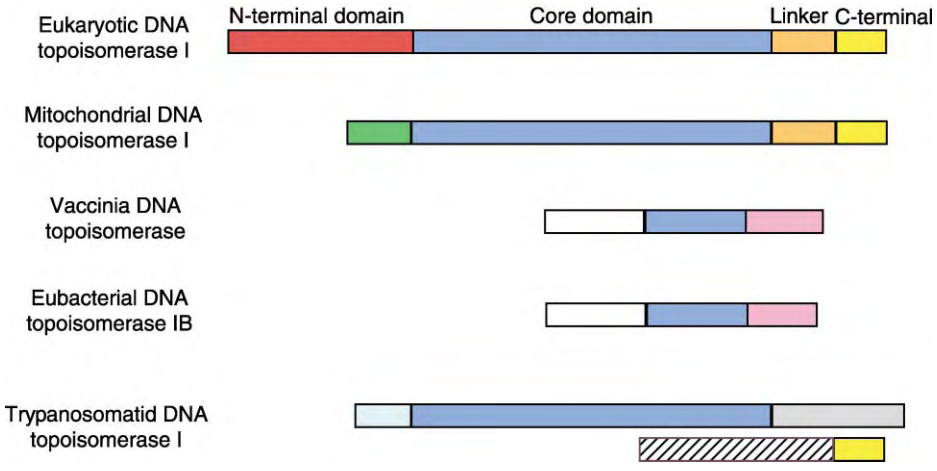



**FIGURE 3** Domain structure and sequence relationships between type IB topoisomerases. The domain structure of the type IB topoisomerases from the indicated sources are denoted by colored boxes with similar domains aligned vertically. The names of the domains for the eukaryotic type IB topoisomerases are shown along the top. Regions that are similar in amino acid sequence share the same color; distinct sequences are assigned different colors. The two subunits of the heterodimeric topoisomerase I from trypanosomatids are shown with these same color conventions.

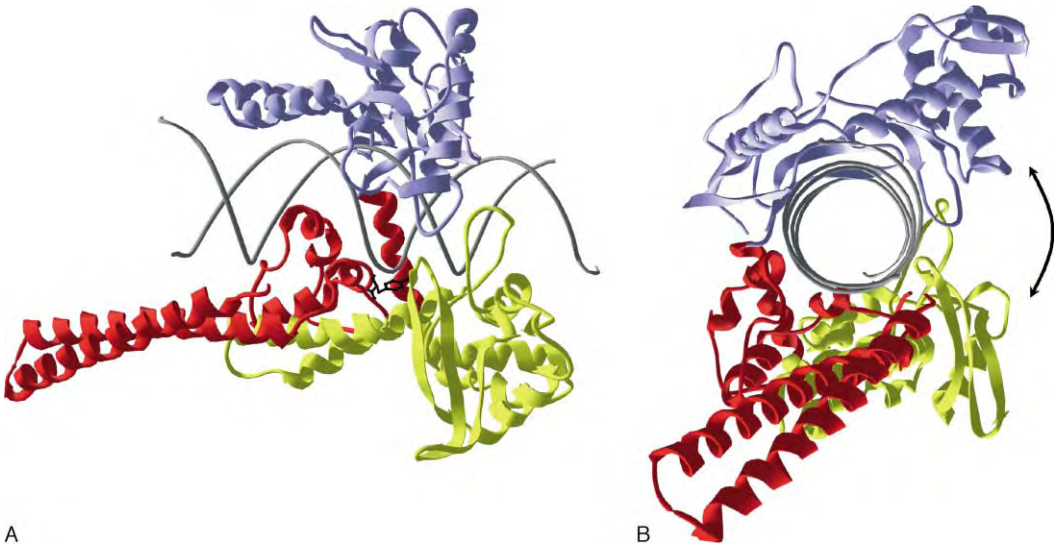



**FIGURE 4** The ribbon diagrams show two views of the crystal structure of human topoisomerase I clamped around a 22 base pair duplex DNA, shown in gray (pdb entry 1a36). The top lobe of the enzyme is shown in blue and the bottom lobe is shown in red and yellow. The poxviral topoisomerase and the eubacterial topoisomerases IB are structurally very similar to the region of the bottom lobe, shown in yellow. The coiled-coil linker region in bottom lobe (in red) is most easily seen in the side view shown in (A). The active site tyrosine (in black) is shown in (A). Panel (B) shows a view of the structure looking down the axis of the DNA. The double-headed arrow in (B) indicates the nature of the conformational change that is required to open and close the clamp during binding and release of the DNA.

the human topoisomerase I structure shared by the poxviral and eubacterial IB enzymes corresponds to the portion of the core domain depicted in yellow in Figure 4. Tyrosine recombinases such as the bacteriophage λ and HP1 integrases, and cre recombinase are also structurally very similar to approximately this same region.

### Catalysis

The co-crystal structure of human topoisomerase I with bound DNA reveals which amino acid residues in the protein are directly involved in catalysis. It is worth noting that the nucleophilic tyrosine O-4 does not appear to be activated for cleavage by general base catalysis, although a lysine residue acts as a general acid to protonate the leaving 5′ oxygen. The pentavalent transition state is stabilized by hydrogen-bonding interactions between three basic amino acid side chains and the scissile phosphate oxygens. An interaction between a lysine residue and the base of the nucleotide where cleavage occurs is also important for catalysis. Religation is likely to proceed by a pathway that is essentially the reverse of cleavage.

### Rotational Mechanism for Strand Passage

Examination of the crystal structure of human topoisomerase I (Figure 4A) suggests that the strand passage reaction required to change the linking number of the DNA during DNA relaxation occurs by a rotational mechanism rather than by the enzyme-bridging mechanism described above for the type IA enzymes. However, there appears to be insufficient space within the confines of the enzyme to accommodate unrestricted rotation of the DNA. This feature of the structure suggests that the enzyme probably undergoes a conformational change after cleavage to open up the space downstream of the cleavage site to allow rotation. Unlike the enzyme-bridging model, a rotational mechanism places no *a priori* limit on the number of rotational events that can occur for each cycle of cleavage and religation. Indeed, in the case of vaccinia topoisomerase, five rotations of the DNA occur on the average between each cleavage and religation reaction.

## Cellular Roles

Although much is yet to be learned about how the various topoisomerases collaborate to manage DNA topology in the cell, a partial picture has emerged based on work in bacteria and simple eukaryotes. Although the type II topoisomerases are not the subject of this review, the activities of these enzymes are briefly considered in the sections to follow for the sake of completeness. The type II enzymes are important for any cellular process that requires the passage of a region of duplex DNA through a double-strand break in the same or a different DNA molecule. The allocation of functions to

the known topoisomerases in setting the global levels of supercoiling, in transcription and in DNA replication, are discussed below.

## Types of Supercoiling in Eukaryotic Versus Prokaryotic Cells

Two different situations lead to the supercoiling of DNA *in vivo*. First, DNA will assume a supercoiled configuration through an interaction with certain proteins or other cellular components. Alternatively, a closed domain of DNA (e.g., a closed circular DNA) will spontaneously supercoil if the linking number is not the same as the helical winding (referred to as twist) of the DNA helix. This latter type of supercoil is often referred to as torsionally strained supercoils. The chromosomal DNA of eukaryotes is wrapped into a protein-constrained solenoidal superhelix in nucleosomes and, except for the transient occurrence of torsionally strained supercoils associated with replication and transcription, is maintained in a relaxed state by DNA topoisomerases. However, in prokaryotes it appears that although some supercoils are constrained by virtue of an interaction with proteins as in eukaryotes, there exists, in addition, a fixed steady-state level of torsionally strained supercoiling generated by gyrases (see below).

## Generation of Supercoiling Stress in Prokaryotes

### Mesophilic Bacteria

The DNA in all mesophilic eubacteria and archaebacteria contains torsionally strained negative supercoils that are introduced by the type II enzyme called DNA gyrase. It appears that this steady-state level of negative superhelicity is required to facilitate helix opening during the initiation of DNA replication and transcription. To prevent the introduction of excess negative supercoils by the gyrase, these bacteria also contain one or two type IA DNA topoisomerases (topoisomerases I or III or both) to counteract the effects of DNA gyrase. The inability of the type IA enzymes to remove negative supercoils below a critical threshold level prevents these enzymes from negating the effects of DNA gyrase and is crucial for fine-tuning the negative supercoiling levels in these organisms. Some eubacteria also contain a type IB enzyme (Table II), which could also balance the effects of DNA gyrase; but the apparent ability of these enzymes to completely relax the DNA suggests that their activity would have to be regulated in some way. The exact role played by the bacterial topoisomerase IB and why this enzyme is only present in a subset of the mesophilic eubacteria remains unknown.

**TABLE II**

**Occurrence of DNA Topoisomerase IB in Eubacteria**

| Known species possessing type IB topoisomerase | Examples of species lacking type IB topoisomerase |
|---|---|
| *Mycobacterium avium* | |
| *Mycobacterium smegmatis* | *Streptomyces coelicolor* |
| *Cytophaga hutchinsonii* | *Chlamydia trachomatis* |
| *Agrobacterium tumefaciens* | *Bacillus anthracis* |
| *Bradyrhizobium japonicum* | *Bacillus subtilis* |
| *Mesorhizobium loti* | *Clostridium tetani* |
| *Sinorhizobium meliloti* | *Mycoplasma pneumoniae* |
| *Rhodobacter sphaeroides* | *Listeria monocytogenes* |
| *Novosphingobium aromaticivorans* | *Staphylococcus aureus* |
| *Bordetella parapertussis* | *Streptococcus pneumoniae* |
| *Burkholderia fungorum* | *Streptococcus pyogenes* |
| *Xanthomonas axonopodis* | *Caulobacter crescentus* |
| *Xanthomonas campestris* | *Rickettsia conorii* |
| *Pseudomonas aeruginosa* | *Neisseria meningitidis* |
| *Pseudomonas fluorescens* | *Helicobacter pylori* |
| *Pseudomonas putida* | *Escherichia coli* |
| *Pseudomonas syringae* | *Yersinia pestis* |
| *Deinococcus radiodurans* | *Vibrio cholerae* |
| | *Xylella fastidiosa* |
| | *Haemophilus influenzae* |
| | *Salmonella typhimurium* |
| | *Borrelia burgdorferi* |
| | *Treponema pallidum* |

### Hyperthermophilic Bacteria

All hyperthermophilic eubacteria and archaebacteria possess a reverse gyrase that actively maintains positive supercoils in the chromosomal DNA. It appears this positive supercoiling is necessary to stabilize the DNA helix against denaturation at the high growth temperatures of these organisms. The mechanism for preventing excess positive supercoiling is not known, but it is likely that a type II enzyme that can relax positive supercoils (DNA gyrase or the archaeal topoisomerase VI) counteracts the effects of reverse gyrase to set the final steady-state level of positive supercoiling.

## Transcription

During transcription, the movement of RNA polymerase along a DNA that is rotationally fixed transiently generates positive supercoils in front of the translocating polymerase and negative supercoils behind the polymerase (Figure 5A). The type IA enzymes present in all organisms relax the negative supercoils that accompany transcription, but the mechanism for the removal of the positive supercoils depends on the organism.

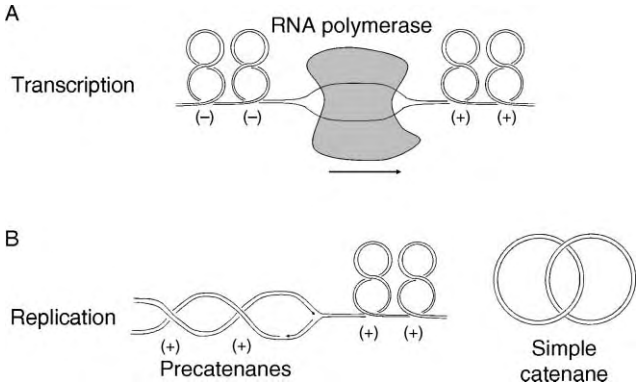



**FIGURE 5** Topological transformations of DNA during transcription and replication. (A) The generation of positive supercoils (+) in front of and negative supercoils (−) behind a translocating RNA polymerase during transcription are depicted. (B) Replication fork movement in a closed domain results in an overwinding of the DNA ahead of the replication fork and the interwinding of the two daughter helices to form precatenanes behind the replication fork, as shown in (B). Any precatenanes remaining at the end of replication for a circular replicon will result in the catenation of the two daughter circular molecules. A simple catenane with a single interlink is also shown.

In prokaryotes, positive supercoils are relaxed by a type II enzyme such as DNA gyrase or in the archaea by topoisomerase VI, and in the case of select eubacteria (see Table II) probably by a combination of a type II enzyme and the type IB topoisomerase.

In eukaryotes, it is likely that the type IB DNA topoisomerase I relaxes the supercoils of both signs associated with transcription. Based on the known complete genome sequences, most eukaryotes, but not fungi or *Caenorhabditis elegans*, contain two distinct genes for type IB enzymes, one for the nuclear enzyme and a second for an enzyme that is imported into the mitochondrion to presumably function in transcription. In those eukaryotic organisms with only a single type IB enzyme, topoisomerase I likely plays a dual role, acting in both the nucleus and the mitochondrion.

## DNA Replication

As the two DNA strands are separated during DNA replication, the DNA helix in front of the replication fork becomes at least transiently overwound or positively supercoiled (Figure 5B). This overwinding of the helix has been shown to be at least partially transmitted to the region behind the replication fork to cause an interwinding of the two daughter helices. These interwindings are referred to as precatenanes (Figure 5B), since in a circular replicon, if any remain at the end of replication, the two daughter circular molecules will be catenated. Resolution of the overwound structure of a replicating chromosome can be accomplished by relaxing positive supercoils in front of the fork, or by decatenating (or unlinking) the precatenanes behind the moving fork, or both.

As in transcription, positive supercoils can be removed in prokaryotes by a type II topoisomerase such as the DNA gyrase, the archaeal topoisomerase VI, or, for those eubacteria that have it, topoisomerase IB. Precatenanes can be resolved by a type II topoisomerase, most notably topoisomerase IV in eubacteria or topoisomerase VI in archaea. As long as gaps exist during discontinuous DNA synthesis, precatenanes can also be unlinked in some eubacteria either by topoisomerase IB (Table II) or by the potent type IA decatenating enzyme, topoisomerase III. Segregation of true catenanes lacking nicks or gaps in either of the two strands can only be accomplished by a type II enzyme.

In eukaryotes, the type IB topoisomerase relaxes the positive supercoils ahead of the replication fork and during synthesis can probably decatenate the precatenanes by acting at gaps in the DNA. However, most of the precatenanes and terminally interlinked or catenated structures are likely resolved by a type II topoisomerase. In mitochondria, replication-associated positive supercoils are relaxed by a type IB topoisomerase as described above for transcription. A mitochondrial type II topoisomerase and a type IA enzyme (topoisomerase III) are likely involved in unlinking precatenanes and catenated circular DNAs that occur during mitochondrial DNA replication.

## See Also the Following Articles

DNA Supercoiling • DNA Topoisomerases: Type II

## Glossary

**catenane** Two interlocked circular DNA molecules in which the two duplexes are wound around each other one or more times.

**catenate** The process whereby two circular DNAs are interlocked to form a catenane. The unlocking of catenated DNAs is referred to as decatenation.

**closed circular DNA** Circular DNA in which both strands are intact.

**DNA gyrase** DNA topoisomerase that couples the hydrolysis of ATP to the introduction of negative supercoils into a closed circular DNA.

**DNA supercoiling** The coiling of the axis of a DNA molecule in three-dimensional space. Supercoiling may result from an interaction of the DNA with protein or from an inequality between the number of helical turns dictated by the structure of the DNA helix under a particular set of conditions (the twist of the DNA) and the linking number of the DNA.

**linking number** Topological property of a closed circular DNA that is a measure of the fixed interwinding of the two DNA strands.

**precatenanes** The interwinding of the two daughter duplexes behind a replication fork.

**reverse gyrase** DNA topoisomerase that couples the hydrolysis of ATP to the introduction of positive supercoils into a closed circular DNA.

**scissile strand** The strand of DNA that is cleaved by a type I topoisomerase.

**topoisomerase** Enzyme that changes the linking number of a closed circular DNA by temporarily breaking one (type I) or both (type II) of the strands of the DNA.

**topoisomers** Variants of a closed circular DNA that have different linking numbers.

**torsionally strained supercoils** Supercoils that result from an inequality between the number of helical turns dictated by the structure of the DNA helix under a particular set of conditions and the linking number of the DNA.

## Further Reading

Alexandrov, A. I., Cozzarelli, N. R., Holmes, V. F., Khodursky, A. B., Peter, B. J., Postow, L., Rybenkov, V., and Vologodskii, A. V. (1999). Mechanisms of separation of the complementary strands of DNA during replication. *Genetica* **106**, 131–140.

Champoux, J. J. (2001). DNA topoisomerases: Structure, function, and mechanism. *Annu. Rev. Biochem.* **70**, 369–413.

Wang, J. C. (1996). DNA topoisomerases. *Annu. Rev. Biochem.* **65**, 635–692.

Wang, J. C. (2002). Cellular roles of DNA topoisomerases: A molecular perspective. *Nat. Rev. Mol. Cell. Biol.* **3**, 430–440.

## Biography

James J. Champoux is a Professor in the Department of Microbiology in the School of Medicine at the University of Washington. His research focuses on topoisomerases and reverse transcription. He holds a Ph.D. from Stanford University and carried out his postdoctoral work at the Salk Institute in San Diego, California. He discovered the eukaryotic type IB topoisomerase and was the first to show that the reaction proceeds through an enzyme–DNA covalent intermediate. He has been instrumental in elucidating the roles of the RNase H activity of reverse transcriptase in retroviral replication.

# DNA Topoisomerases: Type II

**Renier Vélez-Cruz and Neil Osheroff**
*Vanderbilt University School of Medicine, Nashville, Tennessee, USA*

**Although the genetic information of an organism is encoded by the linear array of DNA bases that make up its genome, the three-dimensional properties of the double helix dramatically affect how this information is expressed and passed from generation to generation. Some of the most important three-dimensional relationships in the genetic material are topological in nature, including DNA under- and overwinding, knotting, and tangling. The enzymes that modulate the topological properties of DNA are termed DNA topoisomerases. There are two classes of topoisomerases, type I and type II, which are defined by their reaction mechanisms. Type I topoisomerases alter DNA topology by creating a transient single-stranded break in the genetic material and facilitating controlled rotation of the double helix about (or strand passage through) the nick. Type II topoisomerases act by passing an intact double helix through a transient double-stranded break that they generate in a separate DNA segment. As a consequence of their reaction mechanisms, both classes of enzymes can regulate DNA under- and overwinding. However, because type II topoisomerases cut both strands of the double helix, they also are able to resolve knots and tangles in the genetic material. Type II topoisomerases are essential to all species. Beyond their critical physiological functions, these enzymes are the targets for some of the most important anticancer and antibacterial drugs in clinical use.**

## DNA Topology

The topological properties of DNA are defined as those that cannot be altered without breaking one or both strands of the double helix. Because DNA comprises two interwound nucleic acid strands and the genomes of all known organisms are very long or circular (or both), two distinct topological issues arise as a result of the genetic material. Proliferating cells must be able to cope with both of these in order to survive.

The first issue is related to the torsional stress on the double helix. The DNA from all species of eukaryotes and eubacteria is globally underwound ~5–10%. DNA under torsional stress is termed supercoiled (underwound molecules are negatively supercoiled and overwound molecules are positively supercoiled) because underwound or overwound DNA writhes about itself to form superhelical twists. Negative supercoiling puts energy into the genetic material and makes it easier to separate the two strands of the double helix for replication and transcription. Thus, DNA underwinding dramatically increases the rates of these two fundamental processes. In contrast, the movement of DNA tracking systems (such as replication forks and transcription complexes) through the double helix locally overwinds the DNA ahead of their actions. Because overwinding makes it much harder to pull apart the double helix, it blocks many essential cellular processes.

The second issue is related to the extreme length of genomic DNA. Nucleic acid knots (intramolecular) and tangles (intermolecular) are formed routinely during a variety of ongoing cellular processes including DNA recombination and replication. Both knots and tangles must be resolved in order for daughter chromosomes to segregate properly during meiosis and mitosis.

## DNA Topoisomerases

Cells contain ubiquitous enzymes known as DNA topoisomerases that maintain the appropriate level of DNA supercoiling and remove knots and tangles from the genetic material. These enzymes modulate the topological structure of the genetic material by creating transient breaks in the backbone of DNA. There are two classes of topoisomerases that can be distinguished by the number of DNA strands that they cleave during their catalytic cycles. Type I enzymes create transient single-stranded DNA breaks, whereas type II enzymes create transient double-stranded breaks. To maintain genomic integrity during their DNA cleavage events, topoisomerases form covalent linkages between active-site tyrosyl residues and the newly generated DNA termini. These covalent protein-cleaved DNA complexes, known as cleavage complexes, are the hallmarks of all topoisomerases irrespective of enzyme classification. Because type I topoisomerases create single-stranded breaks in the genetic material, they can regulate DNA supercoiling. However, because type II topoisomerases generate double-stranded breaks in the DNA backbone, they can

 © 2004, Elsevier Inc. All Rights Reserved. 

resolve knots and tangles in addition to removing torsional stress from the genetic material.

Type II topoisomerases are essential to all eukaryotic and prokaryotic organisms. They are highly conserved among species, and the eukaryotic enzymes appear to be direct descendents of ancestral bacterial proteins.

## Eukaryotic Type II Topoisomerases

The eukaryotic type II enzyme is called topoisomerase II. It was discovered in 1980 and is a member of the type IIA homology subfamily. Topoisomerase II can remove positive and negative superhelical twists from the double helix and can resolve DNA knots and tangles.

### Enzyme Mechanism

Topoisomerase II interconverts different topological forms of DNA by the double-stranded DNA passage reaction depicted in Figure 1, which shows the products of each individual step. Briefly, it is proposed that topoisomerase II (1) binds two DNA segments, (2) creates a double-stranded break in one of the segments, (3) translocates the other DNA segment through the cleaved double helix, (4) rejoins (i.e., ligates) the cleaved DNA, (5) releases the translocated segment through a gate in the protein, and (6) closes the protein gate and regains the ability to start a new round of catalysis. The scissile bonds on the two strands of the double helix that are cut by topoisomerase II are staggered. Thus, the enzyme generates cleaved DNA molecules that contain four-base single-stranded ends at their 5′-termini. During its cleavage event, topoisomerase II covalently attaches to these newly generated 5′-termini.

Topoisomerase II requires two cofactors in order to carry out its catalytic double-stranded DNA passage reaction. First, it needs a divalent cation for all steps beyond enzyme-DNA binding (Figure 1, complex 1). Magnesium(II) appears to be the divalent cation that the enzyme uses *in vivo*. Second, topoisomerase II uses the energy of adenosine triphosphate (ATP) to drive the overall DNA strand passage reaction. Although ATP is not required for either DNA cleavage or ligation, the binding of this nucleoside triphosphate triggers DNA translocation (which converts complex 2 to complex 3)

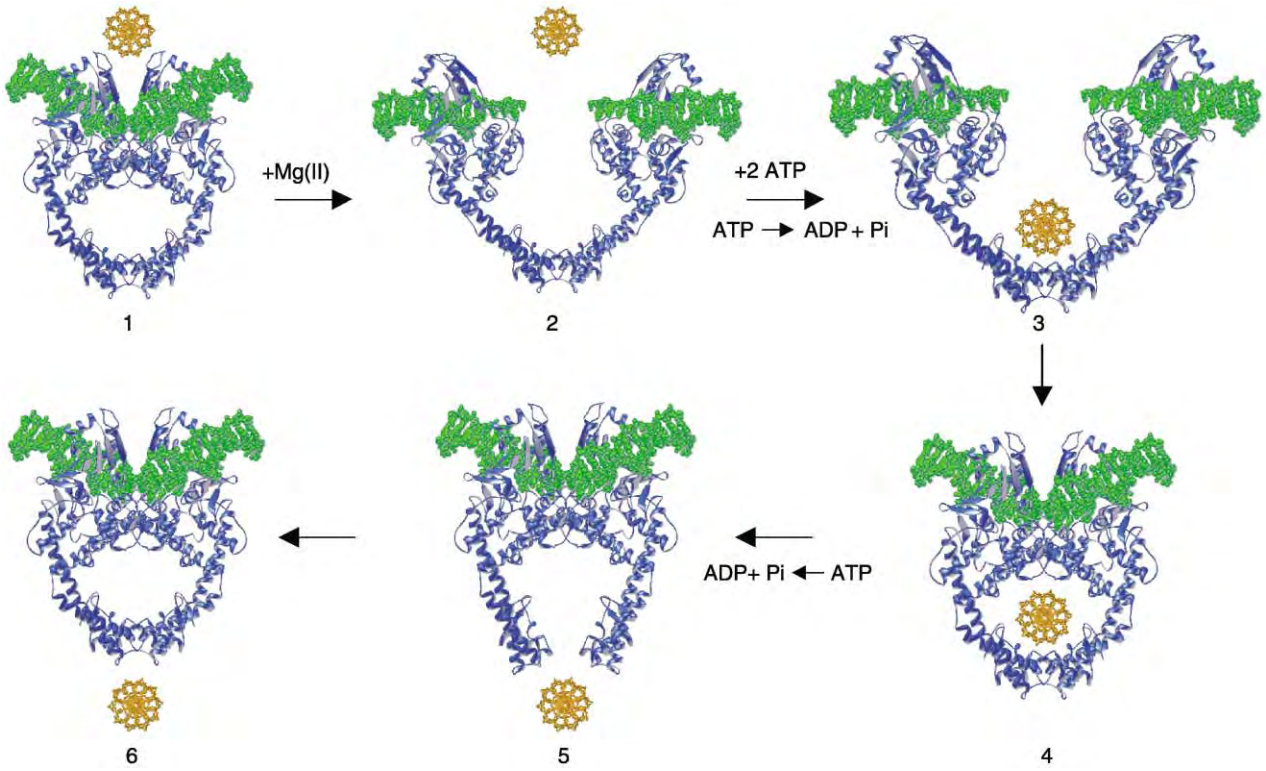



**FIGURE 1** Catalytic cycle of type IIA topoisomerases. The complete double-stranded DNA passage reaction is shown as a series of discrete steps (the products of each step are shown). (1) Enzyme–DNA binding, (2) DNA cleavage (formation of cleavage complex), (3) double-stranded DNA passage, (4) DNA ligation, (5) gate opening and release of the translocated DNA helix, and (6) enzyme recycling. The protein (shown in blue) is based on the crystallographic structure of the catalytic core of yeast topoisomerase II. Modeled DNA helices are shown in green (horizontal) and orange (coming out of the plane of the paper). Structures are courtesy of Dr. James M. Berger, University of California, Berkeley.

and its hydrolysis to adenosine diphosphate (ADP) and inorganic phosphate (Pi) is necessary for enzyme recycling (which converts complex 5 to complex 6). Normally, topoisomerase II binds two molecules of ATP. Although hydrolysis of the cofactor is not a prerequisite for the strand passage event, it appears that this step proceeds more rapidly if it is preceded by hydrolysis of one of the bound ATP molecules.

## Enzyme Domain Structures and Isoforms

Eukaryotic type II topoisomerases are homodimeric enzymes with protomer molecular masses ranging from ~160 to 180 kDa (depending on the species). On the basis of amino-acid-sequence comparisons with the bacterial type II enzyme, DNA gyrase, each enzyme monomer can be divided into three distinct domains (Figure 2). The N-terminal domain of the enzyme is homologous to the B-subunit of DNA gyrase (GyrB) and contains consensus sequences for ATP binding. The central domain is homologous to the A-subunit of DNA gyrase (GyrA) and contains the active-site tyrosyl residue that forms the covalent bond with DNA during scission. The C-terminal domain is not highly conserved and appears to have no corresponding region of homology with DNA gyrase. This variable region of the eukaryotic enzyme contains nuclear localization sequences as well as amino acid residues that are phosphorylated *in vivo*.

Although some eukaryotic species such as yeast and *Drosophila* appear to have only a single type II topoisomerase (i.e., topoisomerase II), vertebrates contain two closely related isoforms, topoisomerase II$\alpha$ and $\beta$. These two isoforms share extensive amino acid sequence identity (~70%), but are encoded by separate genes (located at chromosomal bands 17q21–22 and 3p24 in humans, respectively) and can be distinguished by their protomer molecular masses (~170 and ~180 kDa, respectively).

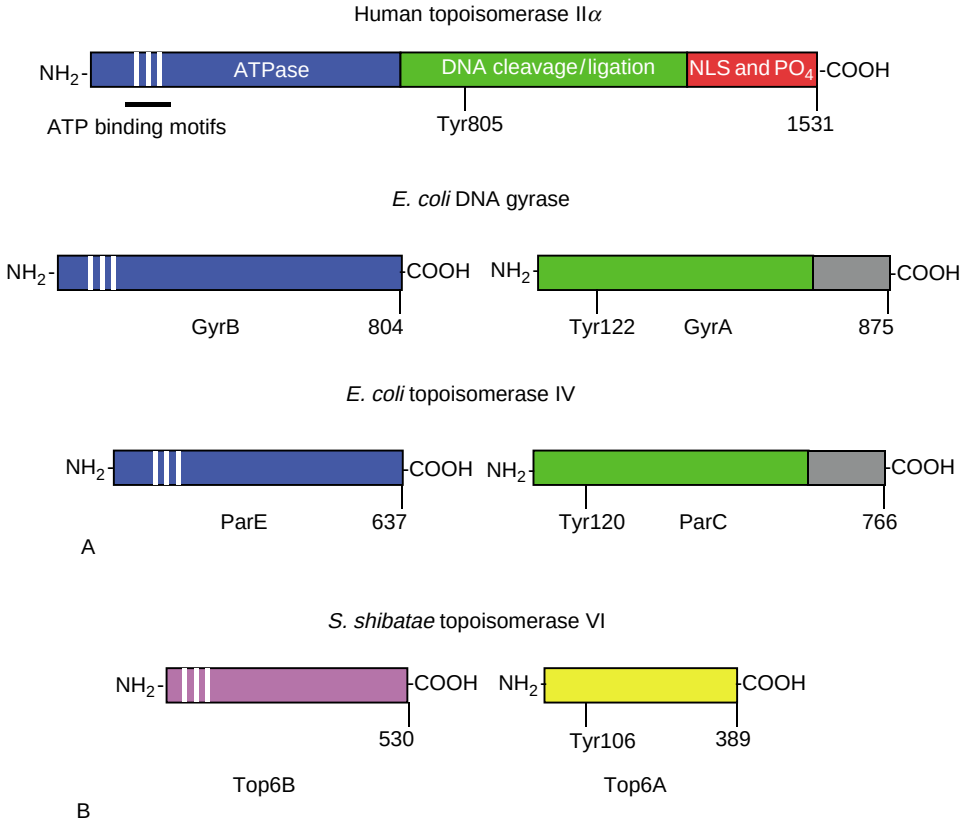



**FIGURE 2** Domain structures of type II topoisomerases. (A) The domain structures of three type IIA topoisomerases: human topoisomerase II$\alpha$ and bacterial (*Escherichia coli*) DNA gyrase and topoisomerase IV. Regions of homology among the enzymes are indicated by colors. The N-terminal (i.e., GyrB) homology domains contain the regions responsible for ATP binding and hydrolysis. The vertical white stripes represent the three conserved motifs of the Bergerat fold that define the ATP-binding domain. The central (i.e., GyrA) homology domains contain the active site tyrosyl residue (Tyr805 in human topoisomerase II$\alpha$) that forms the covalent bond with DNA during scission. For human topoisomerase II$\alpha$, the variable C-terminal domain contains nuclear localization sequences (NLS) and phosphorylation sites ($PO_4$). (B) The Top6A and Top6B subunits of the archaeal type IIB topoisomerase, *Sulfolobus shibatae* topoisomerase VI, shown for comparison.

### Physiological Functions

Topoisomerase II plays a number of essential roles in eukaryotic cells and participates in virtually every major process that involves the genetic material. It unlinks daughter chromosomes that are tangled following replication and resolves DNA knots that are formed during recombination. It also helps to remove the positive DNA supercoils that are generated ahead of replication forks and transcription complexes. Topoisomerase II is required for proper chromosome condensation, cohesion, and segregation and appears to play roles in centromere function and chromatin remodeling. Finally, the type II enzyme is important for the maintenance of proper chromosome organization and structure, and it is the major nonhistone protein of the mitotic chromosome scaffold and the interphase nuclear matrix.

It is not obvious why vertebrate species possess two distinct topoisomerase II isoforms. Enzymological differences between topoisomerase II$\alpha$ and $\beta$ are subtle and the relationships between these isoforms are not well defined. Although either enzyme can complement yeast strains lacking topoisomerase II activity, topoisomerase II$\alpha$ is essential for proliferating mammalian cells and its loss cannot be compensated by the $\beta$ isoform. Topoisomerase II$\beta$ appears to be dispensable at the cellular level, but is required for proper neural development in mice.

The specific cellular functions of topoisomerase II$\alpha$ and $\beta$ probably reflect their physiological regulation more than their enzymological characteristics. Topoisomerase II$\alpha$ is regulated over both cell and growth cycles. Enzyme levels increase throughout the S-phase of the cell cycle and peak at the $G_2$-M boundary. Furthermore, this isoform is found almost exclusively in rapidly proliferating tissues. In contrast, the concentration of topoisomerase II$\beta$ is independent of the cell cycle and this isoform is found in most cell types regardless of proliferation status. Taken together, these characteristics suggest that topoisomerase II$\alpha$ is the isoform responsible for events associated with DNA replication and chromosome segregation, whereas topoisomerase II$\beta$ is the isoform that probably functions in ongoing nuclear processes.

## Prokaryotic Type II Topoisomerases

Eubacteria contain two distinct type II topoisomerases, DNA gyrase and topoisomerase IV. Both are members of the type IIA subfamily. In addition to these two enzymes, many archaeal species contain a third type II enzyme, topoisomerase VI. This last enzyme is a member of the type IIB subfamily.

### DNA Gyrase

DNA gyrase was discovered in 1976. It was the first type II topoisomerase to be described and is the only one to retain its historical name (in the modern nomenclature, type II topoisomerases are denoted by even numbers). In contrast to the eukaryotic type II enzymes, DNA gyrase is comprised of two distinct subunits, GyrA and GyrB (molecular mass $\approx$96 kDa and 88 kDa, respectively) and is arranged as an $A_2B_2$ tetramer. GyrA contains the active site tyrosine used in DNA cleavage and ligation, and GyrB contains the binding site for ATP (Figure 2A).

In contrast to all other type II topoisomerases, DNA gyrase is the only enzyme that is capable of actively underwinding (i.e., negatively supercoiling) the double helix. It accomplishes this feat by wrapping DNA around itself in a right-handed fashion and carrying out its strand-passage reaction in a unidirectional manner.

The negative supercoiling activity of DNA gyrase far exceeds the ability of the enzyme to remove either knots or tangles from the genetic material. Consequently, the major physiological roles of DNA gyrase stem directly from its ability to underwind the double helix. DNA gyrase plays a critical role in opening DNA replication origins and removing positive supercoils that accumulate in front of replication forks and transcription complexes. In addition, this enzyme works in conjunction with the ω protein (a type I topoisomerase that removes negative supercoils from the double helix) to maintain the global balance of DNA supercoiling in bacterial cells.

### Topoisomerase IV

Topoisomerase IV is an $A_2B_2$ tetramer that is comprised of two distinct subunits, ParC (molecular mass $\approx$88 kDa), and ParE (molecular mass $\approx$70 kDa), which are homologous to the A- and B-subunits of DNA gyrase (Figure 2B). (In gram-positive bacterial species, the subunits of topoisomerase IV are designated GrlA and GrlB, respectively.) It was known for several years that the ParC and ParE proteins were necessary for proper chromosome segregation in bacteria. However, it was not discovered until 1990 that these two subunits together constituted a type II topoisomerase.

The catalytic properties of topoisomerase IV can be distinguished from those of DNA gyrase in two important ways. First, although topoisomerase IV can remove positive and negative superhelical twists from DNA, it cannot actively underwind the double helix. Second, the ability of topoisomerase IV to resolve DNA knots and tangles is dramatically better than that of DNA gyrase. Because of these differences, the physiological roles of topoisomerase IV are distinct from those of DNA gyrase. The primary cellular functions of topoisomerase IV are to unlink daughter chromosomes following DNA replication and to resolve DNA knots

that are formed during recombination. Recently, it was found that topoisomerase IV removes positive supercoils from DNA more efficiently than it removes negative supercoils. This has led to speculation that the enzyme also may act ahead of DNA tracking systems to alleviate overwinding of the double helix. However, the precise role of topoisomerase IV in this process has yet to be defined.

### Archaeal Topoisomerase VI

In 1997, a novel type II topoisomerase, topoisomerase VI, was discovered in hyperthermophilic archaeal species. This enzyme was designated as the first member of the topoisomerase IIB subfamily due to its lack of homology to previously identified type II enzymes.

Topoisomerase VI has two subunits, Top6A and Top6B (molecular masses ≈47 and 60 kDa, respectively), and is arranged as an $A_2B_2$ tetramer. Both subunits are considerably smaller than those of bacterial DNA gyrase or topoisomerase IV (Figure 2B). Although short regions of Top6B surrounding the ATP-binding domain are homologous to portions of GyrB, and Top6A contains an active-site tyrosine that is required for DNA cleavage, the primary structure of topoisomerase VI displays little similarity to the type IIA enzymes.

Archaeal topoisomerase VI appears to alter DNA topology by using a double-stranded DNA passage reaction like that described for other type II topoisomerases. During this reaction, it generates DNA breaks with 5′ overhangs that are covalently attached to its active-site tyrosyl residues. Topoisomerase VI relaxes positively and negatively supercoiled DNA, but cannot actively underwind the double helix. In addition, it can unlink (i.e., untangle) interwound double-stranded DNA circles.

The catalytic properties of topoisomerase VI differ from those of the type IIA enzymes in two significant aspects. First, topoisomerase VI requires ATP binding in order to cleave its DNA substrate. Second, in marked contrast to the type IIA enzymes (which produce four-base staggered ends during scission), topoisomerase VI-mediated DNA cleavage generates DNA termini that contain only two-base overhangs. Although the physiological functions of topoisomerase VI have yet to be determined, the enzyme is believed to play a role in unlinking daughter chromosomes following replication in archaeal cells.

With the exception of plants, no Top6B homologue has been identified in eukaryotic species. However, a Top6A homologue, Spo11, has been found in eukaryotes ranging from yeast to humans. Spo11 generates the double-stranded DNA breaks that initiate meiotic recombination. Like its topoisomerase relatives, Spo11 forms a covalent bond between an active-site tyrosyl residue and the 5′-DNA termini generated by its scission reaction. At the present time there is no evidence that Spo11 has topoisomerase (i.e., DNA strand passage) activity.

## Type II Topoisomerases as Therapeutic Targets

In addition to their varied and critical physiological functions, the type IIA topoisomerases are targets for some of the most active anticancer and antibacterial drugs in clinical use. In contrast to most enzyme-targeted drugs, these agents do not act by robbing cells of an essential enzyme activity. Rather, drugs that target type II topoisomerases kill cells by dramatically increasing the concentration of covalent enzyme-cleaved DNA complexes (i.e., cleavage complexes) that are requisite intermediates formed during the double-stranded DNA passage reaction. Normally, cleavage complexes are present at low steady-state levels and are tolerated by cells. However, conditions that significantly increase either their concentration or lifetime trigger numerous mutagenic events.

The potential lethality of cleavage complexes rises dramatically when DNA tracking enzymes such as polymerases or helicases attempt to traverse the covalently bound topoisomerase roadblock in the genetic material. Such an action disrupts cleavage complexes and converts transient enzyme-mediated DNA breaks to permanent DNA breaks. These permanent breaks in the genome trigger the generation of chromosomal insertions, deletions, translocations, and other aberrations, and, when present in sufficient numbers, they initiate a series of events that culminates in cell death. Because the drugs that target type II topoisomerases convert these essential enzymes to potent cellular toxins that fragment the genome, they are referred to as topoisomerase poisons to distinguish them from drugs that act as catalytic inhibitors.

### Anticancer Drugs

At the present time, six topoisomerase II-targeted anticancer agents (Figure 3A) are approved for use in the United States. Drugs such as etoposide and doxorubicin are front-line therapy for breast and lung cancers, as well as for a variety of leukemias, lymphomas, and germ-line malignancies. Approximately one-half of all cancer chemotherapy regimens contain drugs targeted to topoisomerase II. Moreover, every form of cancer that can be cured by systemic chemotherapy is treated with these agents.

Due to the high concentration of topoisomerase II$\alpha$ in rapidly proliferating cells, this isoform probably is the major important target of anticancer therapy. However, circumstantial evidence suggests that the $\beta$-isoform also contributes to drug efficacy.

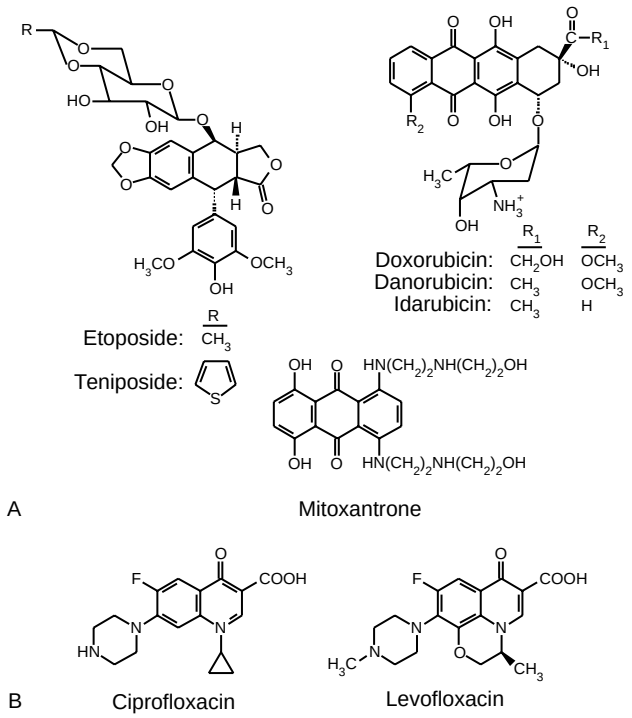



**FIGURE 3** Structures of selected (A) anticancer drugs targeted to topoisomerase II and (B) antibacterial drugs targeted to DNA gyrase and topoisomerase IV.

## Antibacterial Drugs

DNA gyrase and topoisomerase IV are the targets for quinolone-based antibacterial agents (Figure 3B). Quinolones are the most active and broad-spectrum antibacterial drugs currently available. Drugs such as ciprofloxacin are prescribed routinely for a wide variety of gram-negative bacterial infections, including gastrointestinal tract, respiratory tract, and bone and joint infections. Ciprofloxacin also is used to treat a number of sexually transmitted diseases as well as infection with anthrax. Newly developed quinolones, such as levofloxacin, display significant efficacy against gram-positive bacterial infections.

DNA gyrase is the primary cytotoxic target of quinolones in gram-negative bacteria. However, topoisomerase IV appears to be the more important target for many of these drugs in gram-positive species.

## See Also the Following Articles

DNA Supercoiling • DNA Topoisomerases: Type I

## Glossary

**adenosine triphosphate (ATP)** A cofactor that supplies energy for many enzymatic processes.

**cell cycle** The process by which a cell grows, replicates its genome, and divides. The cell cycle is divided in four distinct phases: $G_1$, a growth phase; S, the phase in which the cell duplicates (i.e., synthesizes) its genetic material; $G_2$, a second growth phase in which the cell prepares to divide; and M, the phase in which the cell divides (i.e., mitosis).

**DNA recombination** The process by which the cell reorganizes its genetic material in order to repair certain forms of DNA damage (including double-stranded DNA breaks) or promote genetic diversity.

**DNA replication** The process by which the cell duplicates (i.e., synthesizes) its genetic material.

**DNA supercoiling** The underwinding (i.e., negative supercoiling) or overwinding (i.e., positive supercoiling) of the genetic material.

**topoisomerase poison** A drug that increases levels of topoisomerase-cleaved DNA complexes.

**topology** A field of mathematics that deals with relationships that are not altered by elastic deformation.

**transcription** The process by which the cell expresses its genetic material; the generation of messenger RNAs from a DNA template.

## Further Reading

Anderson, V. E., and Osheroff, N. (2001). Type II topoisomerases as targets for quinolone antibacterials: Turning Dr. Jekyll into Mr. Hyde. *Curr. Pharm. Des.* **7**, 337–353.

Champoux, J. J. (2001). DNA topoisomerases: Structure, function, and mechanism. *Annu. Rev. Biochem.* **70**, 369–413.

Fortune, J. M., and Osheroff, N. (2000). Topoisomerase II as a target for anticancer drugs: When enzymes stop being nice. *Prog. Nucl. Acid Res. Mol. Biol.* **64**, 221–253.

Gadelle, D., Filee, J., Buhler, C., and Forterre, P. (2003). Phylogenomics of type II DNA topoisomerases. *BioEssays* **25**, 232–242.

Heddle, J. G., Barnard, F. M., Wentzell, L. M., and Maxwell, A. (2000). The interaction of drugs with DNA gyrase: A model for the molecular basis of quinolone action. *Nucleosides Nucleotides Nucleic Acids* **19**, 1249–1264.

Li, T. K., and Liu, L. F. (2001). Tumor cell death induced by topoisomerase-targeting drugs. *Annu. Rev. Pharmacol. Toxicol.* **41**, 53–77.

Osheroff, N (ed.) (1998). DNA topoisomerases. *Biochim. Biophys. Acta* **1400**.

Wang, J. C. (2002). Cellular roles of DNA topoisomerases: A molecular perspective. *Nat. Rev. Mol. Cell. Biol.* **3**, 430–440.

## Biography

Renier Vélez-Cruz is completing his doctoral studies in the Department of Biochemistry, Vanderbilt University School of Medicine.

Neil Osheroff is a Professor in the Departments of Biochemistry and Medicine at the Vanderbilt University School of Medicine and holds the John G. Coniglio Chair in Biochemistry. His principal research interests are the fields of DNA topoisomerases, topoisomerase-targeted drugs, and DNA repair. He holds a Ph.D. in Biochemistry and Molecular Biology from Northwestern University and received his postdoctoral training in the Department of Biochemistry at the Stanford University School of Medicine. He has authored more than 170 articles and has contributed significantly to our understanding of the mechanism of action of type II topoisomerases and topoisomerase II poisons.

# DNA Topoisomerases: Type III–RecQ Helicase Systems

**Rodney Rothstein and Erika Shor**
*Columbia University College of Physicians and Surgeons, New York, USA*

**DNA helicases and topoisomerases belong to the category of proteins that physically manipulate and alter the structure of DNA molecules. DNA helicases are enzymes that separate the strands of double-stranded (ds) DNA molecules, thus catalyzing DNA unwinding. Topoisomerases transiently create breaks in a DNA strand(s), pass other strands through the broken strand(s), and reseal the breaks. Topoisomerase activity can change levels of DNA supercoiling or result in catenation (interlinking) or decatenation (unlinking) of two DNA molecules. Both DNA helicases and topoisomerases are key players in various DNA transactions, such as replication, transcription, and recombination. Different classes and families of helicases and topoisomerases have been identified based on their protein sequence conservation, substrate preference, directionality on DNA, and other properties. DNA helicases of the RecQ family have garnered much interest lately because of the involvement of three human RecQ helicase family members in genetic disorders characterized by genomic instability and cancer predisposition. RecQ helicases are evolutionarily conserved proteins found in organisms ranging from bacteria to humans. Interestingly, an association between RecQ-type helicases and type III topoisomerases has been observed throughout the evolutionary tree, suggesting that these two proteins act in concert to promote genomic stability.**

## Structure and Molecular Mechanisms

### Topoisomerase III

Topoisomerase III belongs to the type IA topoisomerases (also known as type I-5′). This subfamily of topoisomerases acts on DNA that is negatively supercoiled (underwound) and/or contains single-stranded (ss) regions. The topoisomerase, which functions as a monomer, makes a break in a ssDNA region via a transesterification reaction between an active site tyrosine of the enzyme and a DNA phosphate group (Figure 1). A transient covalent linkage between the tyrosine and the 5′-phosphoryl group of the DNA is thus formed. After passage of other DNA strand(s) through the break, the reverse transesterification reaction leads to the rejoining of the DNA backbone. Topoisomerase III activity can result in relaxation/removal of negative supercoiling from DNA, (de)catenation and knotting of ss circular DNA molecules, and (de)catenation of ds DNA molecules that contain ss regions. Topoisomerase III does not require energy in the form of nucleoside triphosphates, such as ATP. Hence, the directionality of the topoisomerase III-driven reactions is toward the DNA conformation with the lowest free energy.

### RecQ Helicases

The RecQ family of DNA helicases is defined by homology to the bacterial RecQ protein. Like other helicases, these proteins contain seven signature helicase motifs, including sequences that contain Walker A (required for ATP binding and hydrolysis) and B boxes. In addition to the core helicase motifs, all RecQ helicases share additional regions of homology not shared by other families of helicases.

The functional unit of a helicase is generally composed of a dimer or a hexamer that forms a ring around its substrate DNA. However, examples of monomeric helicases are also known. Whereas several early studies have indicated that RecQ-like helicases form hexamers, two recent studies suggest that DNA helicase activities *in vitro* of both *E. coli* RecQ and human BLM proteins are associated with a monomeric form of the protein. Thus, the composition of a functional unit of RecQ-like helicases is still being explored.

All RecQ-type helicases examined to date display $3' \rightarrow 5'$ directionality on DNA with respect to the strand to which the protein is bound (Figure 2A). *In vitro* substrate preference studies have indicated that these helicases can act on a variety of DNA structures, possibly reflecting the diversity of their *in vivo* activities. Among the structures that RecQ helicases can unwind are branched molecules, four-way dsDNA junctions, G-quartets, and D-loops (Figure 2B). ATP and $Mg^{2++}$ are necessary cofactors for RecQ-driven reactions.

 © 2004, Elsevier Inc. All Rights Reserved. 

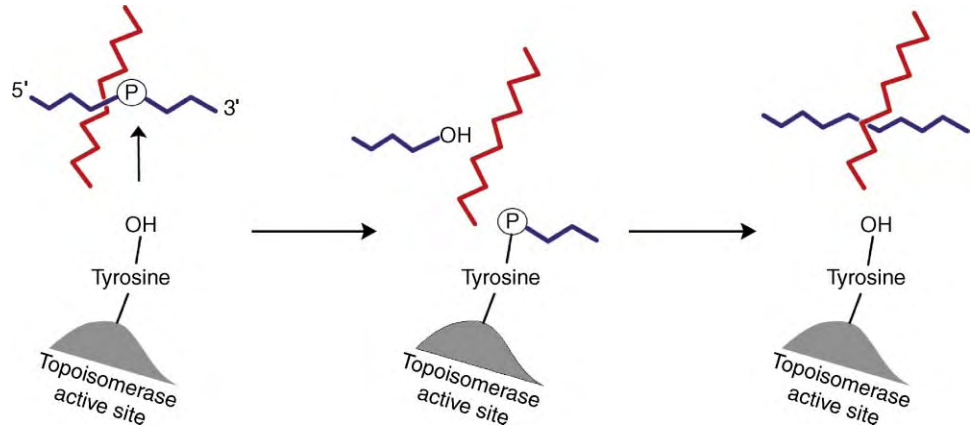



**FIGURE 1** The mechanism of strand passage by a type IA topoisomerase. The topoisomerase makes a break in the blue strand by catalyzing a trans-esterification reaction between the tyrosine at the topoisomerase active site and a 5′ phosphate group of the DNA. Only the reacting phosphate is depicted as a P for clarity. The intact red strand is passed through the break, and a reverse trans-esterification reaction reseals the blue strand.

## The RecQ Helicase–Topoisomerase III Connection

### Physical Association

Two widely used model organisms, budding and fission yeast, each has one RecQ-like helicase and one topo III. In both organisms, the two proteins have been shown to physically interact with each other and/or to associate in cellular extracts. In human cells, the RecQ homolog BLM physically interacts with an isoform of topoisomerase III, Top3$\alpha$, via the N-terminus of BLM. Mutation or deletion of BLM in human cells has several detrimental consequences on genome stability, including a marked increase in recombination between sister chromatids. Interestingly, cells expressing BLM protein that lacks its N-terminus and fails to interact with Top3$\alpha$ (but retains DNA unwinding activity) show increased levels of sister chromatid exchange, similar to cells that lack the entire BLM protein. This demonstrates that, in order to perform its role in suppressing recombination between sister chromatids, BLM needs to interact with Top3$\alpha$ *in vivo*, which supports other evidence that the two act as a complex with key roles in maintenance of genome stability.

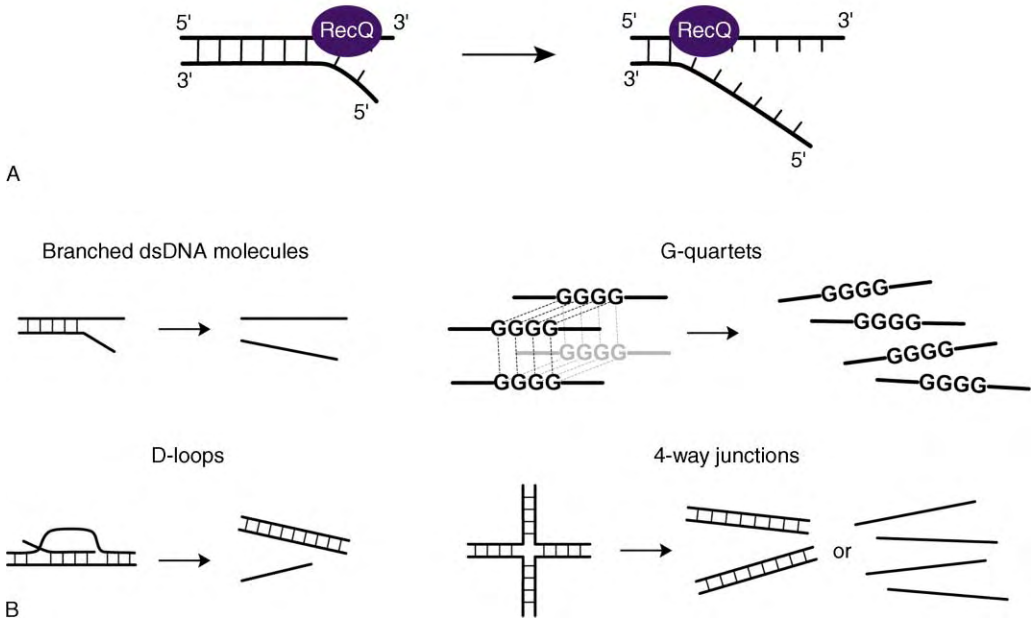



**FIGURE 2** (A) RecQ helicases unwind DNA in the 3′ to 5′ direction with respect to the strand to which the helicase is bound. (B) Several of the known *in vitro* substrates of RecQ helicases before and after incubation with the protein.

### Genetic Interactions in Yeast

As mentioned above, the genomes of both budding and fission yeast encode a single topoisomerase III gene. In both organisms, loss or mutation of the topo III protein is detrimental, resulting in slow growth or lethality. Concomitant loss of RecQ helicase function in mutants lacking topo III largely restores normal growth and viability. These genetic observations have led to the following model of RecQ–Topoisomerase III interaction *in vivo*. It is proposed that in wild-type cells, the activity of the RecQ helicase creates a DNA structure that is normally acted on and resolved by topo III. When topo III is inactivated, this structure is not processed properly and causes slow growth or lethality. In mutants lacking the RecQ helicase, this structure is not created, eliminating the need for a functional topo III. While this model is supported by genetic evidence, experimental data confirming the existence of such a DNA structure are lacking to date.

### Combined Mode of Action

The genetic and physical interactions between RecQ-like helicases and type III topoisomerases in several organisms have prompted investigation and speculation regarding their combined mode of action. One possibility first put forth upon the discovery of the budding yeast RecQ homolog Sgs1 and its genetic and physical association with the budding yeast topo III was that the two proteins form a complex that functions like a eukaryotic reverse gyrase. A reverse gyrase is an enzyme found in several species of *Archaea*. A hallmark feature of the reverse gyrase protein is the presence of a type I topoisomerase-like domain and a helicase domain as part of the same polypeptide. *In vitro*, the enzyme introduces positive supercoiling into dsDNA molecules. This is thought to happen in the following manner. The helicase domain of reverse gyrase unwinds a region of dsDNA, creating local negative supercoiling on one side of the enzyme and local positive superoiling on the other. The topoisomerase domain relaxes the negative supercoiling, resulting in a net overall increase in positive supercoiling in the dsDNA molecule. However, despite the attractiveness of the hypothesis that the RecQ–topoIII complex resembles reverse gyrase, the few *in vitro* studies done on RecQ and topo III have not reported increased positive supercoiling as a result of the combined action of the two enzymes.

Another hypothesis regarding the role of the RecQ–topoIII complex suggests a role in catenation or decatenation of ds DNA molecules. This idea is supported by a study that examined the consequences of concerted action of bacterial RecQ and topo III in an *in vitro* system. It was shown that both bacterial and yeast topo III proteins are specifically stimulated by the bacterial RecQ helicase to fully catenate or decatenate (depending on the conditions of the reaction) covalently closed circular dsDNA molecules. *In vivo*, a decatenation activity may be used to separate such interlinked dsDNA substrates as sister chromatids following DNA replication or homologous chromosomes following mitotic or meiotic recombination. Their complete decatenation is essential for subsequent faithful chromosomal segregation, which in turn ensures accurate transfer of genetic material into daughter cells. On the other hand, a catenating activity may be used *in vivo* to suppress recombination, as DNA sequences that are highly catenated undergo fewer recombination events.

## Cellular Roles of the RecQ–TopoIII Complex in DNA Metabolism

### The Recombination Connection

Inactivation of a RecQ-type helicase or topoisomerase III generally causes a variety of detrimental consequences on the genomic integrity of an organism. One consequence that is shared by all organisms examined to date is an increase in genetic recombination. As mentioned above, hyper-recombination between sister chromatids is a hallmark of human cells expressing mutant versions of BLM protein. In budding yeast, inactivation of the RecQ homolog Sgs1 or of the topo III homolog Top3 leads to an increase in several kinds of recombination, such as recombination between tandemly repeated DNA sequences. In *E. coli*, inactivation of the *recQ* gene leads to an increase in illegitimate recombination (i.e., recombination between nonhomologous DNA sequences). These observations have suggested that RecQ helicases generally control or suppress recombination. This idea is supported by a series of genetic experiments with budding yeast and mirrored by observations in fission yeast. In these organisms, concomitant mutation of the RecQ family member and of another helicase, Srs2, causes extreme slow growth and frequent lethality. However, these defects are fully rescued by mutation of any of several proteins that perform homologous recombination. This indicates that in the absence of a functional homologous recombination pathway, deletion of the two helicases is no longer detrimental to the cell. This can be explained by proposing that in the absence of the two helicases, incorrectly regulated or uncontrolled recombination is responsible for the poor growth and increased lethality. Physical interactions between RecQ helicases and proteins that function in recombination add further support to the connection between the RecQ family and recombination. In both yeast and humans, a RecQ

family member was shown to physically interact with Rad51, a protein that performs key steps in homologous recombination, such as the invasion of a DNA duplex by a homologous single strand and subsequent branch migration. Also, biochemical activities of several RecQ helicases are consistent with a direct role in suppressing recombination. Human BLM and yeast Sgs1 proteins efficiently unwind or branch-migrate four way DNA junctions *in vitro* (Figure 2B). Interestingly, four-way junctions closely resemble the structure of an intermediate in genetic recombination, a Holliday junction. Another human RecQ homolog, WRN, unwinds D-loops, DNA intermediates formed by the first step of homologous recombination—the strand invasion of a duplex by a ssDNA molecule (Figure 2B). Thus, RecQ helicases may control recombination *in vivo* by disrupting recombination intermediates.

### The Replication Connection

Several lines of evidence indicate that RecQ-like helicases and topo III function during DNA replication. Analyses of mRNA and protein levels of the budding yeast RecQ and topo III homologs have shown that expression of these genes fluctuates throughout the cell cycle, peaking during and right after the period of active DNA replication. Also, yeast mutants lacking the RecQ helicase or topo III are sensitive to chemicals that arrest DNA replication by depleting cellular pools of deoxynucleotide triphosphates (dNTP's)—the building blocks necessary to assemble new DNA molecules. For instance, wild-type fission yeast cells are able to resume normal replication after transient exposure to one such chemical, hydroxyurea, whereas mutants that lack the RecQ helicase fail to make the recovery. These observations indicate that the function of RecQ family proteins is especially important for cellular survival if DNA replication is stalled.

Whereas chemicals such as hydroxyurea can arrest the progress of DNA replication throughout the entire genome, occasional pausing in the progression of individual replication forks is thought to occur spontaneously in a high proportion of cells. Accordingly, proteins that are necessary for proper handling and eventual restart of these paused forks are required throughout DNA replication even in cells unchallenged by exogenous agents. Consistent with a role for RecQ helicases during normal cell cycle, analysis of human cells expressing mutant versions of BLM or WRN proteins has shown that these cells exhibit delayed replication progression and accumulate abnormal replication intermediates.

There are several non-mutually exclusive possibilities for the roles of RecQ family members during DNA replication. These proteins could physically manipulate or maintain replication fork structure. The importance of RecQ helicases for normal progression of DNA replication combined with their previously discussed roles in controlling recombination have led to the idea that these proteins function specifically to suppress recombination initiated at stalled replication forks. Also, RecQ helicases may be involved in replicating "difficult" regions of the genome, such as telomeres. Telomeric DNA is very GC-rich and may form alternative conformations, such as G-quartets (Figure 2B). Several RecQ homologs can unwind G-quartet DNA *in vitro*, and yeast RecQ homolog Sgs1 has been implicated in telomere maintenance *in vivo*. Alternatively, these proteins could detect fork stalling and signal to other molecules, resulting in recruitment of factors necessary for resumption of fork movement. Indeed, recent evidence suggests that some RecQ family members may be involved in DNA damage surveillance and signaling mechanisms called checkpoints. In particular, the budding yeast RecQ homolog Sgs1 has been shown to participate in such signaling mechanisms specific to DNA damage occurring during DNA replication.

## RecQ Helicases and Human Disease

The human genome encodes five proteins that belong to the RecQ class of helicases. Mutations in three of these proteins, BLM, WRN, and RECQ4, cause genetic disorders: Bloom, Werner, and (at least a subset of) Rothmund–Thomson syndromes, respectively. Among other symptoms, Bloom syndrome patients exhibit short stature, immunodeficiency, impaired fertility, and a predisposition to a variety of cancers. At the cellular level, the syndrome is characterized by genomic instability, including hyper-recombination between sister-chromatids and homologous chromosomes. Werner and Rothmund-Thomson syndrome patients exhibit symptoms of premature aging, as well as a predisposition to certain types of cancer. Both syndromes are also characterized by increased genomic instability at the cellular level. Cells cultured from Werner syndrome patients exhibit an increase in illegitimate recombination, resulting in chromosomal deletions and translocations. Less is known about the cellular characteristics of the Rothmund–Thomson syndrome, but these cells also display increased chromosomal abnormalities.

Whereas no genetic disorder is known to result from mutation of a human topo III homolog, mouse knock-out strains have provided important information about the role of these proteins in higher eukaryotes. Deletion of the *TOP3α* gene, encoding one of the two isoforms of topo III in mice and humans, results in embryonic lethality, indicating that this protein has essential functions during development. Deletion of the other topo III isoform, *TOP3β*, does not result in lethality, but

causes a decrease in lifespan, reduced fertility, and increased incidence of aneuploidy. Thus, topo III-like proteins, similar to RecQ helicases, play important roles in mammalian development, aging, and chromosomal integrity.

## See Also the Following Articles

DNA Helicases: Dimeric Enzyme Action • DNA Helicases: Hexameric Enzyme Action • DNA Replication Fork, Eukaryotic • DNA Supercoiling • Glutamate Receptors, Ionotropic

## Glossary

**DNA recombination** Exchange or transfer of genetic material between two DNA molecules, such as two chromosomes in the cell.

**DNA replication** The process of faithful copying of genetic information in a cellular genome prior to cell division. This is accomplished by separating the strands of duplex chromosomal DNA and synthesizing new DNA strands that are complementary to the parental strands.

**replication fork** Y-shaped DNA structure formed during DNA synthesis when the parental DNA strands are separated to provide a template for DNA replication.

**sister chromatids** The identical copies of a single chromosome produced after DNA replication.

**supercoiling** The topological state achieved by twisting a duplex DNA molecule around its axis.

**telomere** Region of DNA at the end of a linear chromosome.

## Further Reading

Champoux, J. J. (2001). DNA topoisomerases: Structure, function, and mechanism. *Annu. Rev. Biochem.* **70**, 369–413.

Lohman, T. M., and Bjornson, K. P. (1996). Mechanisms of helicase-catalyzed DNA unwinding. *Annu. Rev. Biochem.* **65**, 169–214.

Lombard, D. B. (2001). *Biochemistry and Genetics of RecQ-Helicases.* Kluwer Academic, Boston.

Oakley, T. J., and Hickson, I. D. (2002). Defending genome integrity during S-phase: Putative roles for RecQ helicases and topoisomerase III. *DNA Repair* **1**, 175–207.

van Brabant, A. J., Stan, R., and Ellis, N. A. (2000). DNA helicases, genomic instability, and human genetic disease. *Annu. Rev. Genomics Hum. Genet.* **1**, 409–459.

Wang, J. C. (2002). Cellular roles of DNA topoisomerases: A molecular prospective. *Nature Rev.* **3**, 430–440.

## Biography

Rodney Rothstein is a Professor of Genetics and Development at Columbia University College of Physicians and Surgeons in New York. His principal research interests are in the mechanisms of DNA recombination and the cellular response to DNA damage. He holds a Ph.D. from The University of Chicago and received postdoctoral training at the University of Rochester and Cornell University. He developed one-step gene disruption in yeast and is one of the authors of the double-strand break repair model. His laboratory discovered the first eukaryotic topoisomerase III gene family member and the first eukaryotic RecQ homolog, Sgs1.

Erika Shor, a senior graduate student in the Rothstein laboratory, studies the budding yeast RecQ and topoisomerase III family members.

# Dopamine Receptors

**Kim A. Neve**
*VA Medical Center, Portland, Oregon, USA*

**Dopamine receptors are rhodopsin-like seven transmembrane receptors (also called G protein-coupled receptors) that mediate the central and peripheral actions of dopamine. Dopamine receptors are most abundant in pituitary and brain, particularly in the basal forebrain, and are also found in the retina and peripheral organs such as the kidney. Stimulation of dopamine receptors modulates excretion of sodium by the kidney, and both cell division and hormone synthesis and secretion in the pituitary. Brain dopamine receptors regulate movement and locomotion, motivation, and working memory. Five subtypes of mammalian dopamine receptors have been identified that are divided into D1-like (D1, D5) or D2-like (D2, D3, D4) groups. The D1-like receptors couple primarily to the $G_{\alpha s}$ family of G proteins ($G_{\alpha s}$ and $G_{\alpha olf}$), whereas the D2-like receptors couple primarily to the $G_{\alpha i/o}$ family. Drugs that block D2 receptors are useful for the treatment of schizophrenia and other psychoses, while drugs that stimulate D1-like or D2-like receptors alleviate the motor symptoms that result from degeneration of dopamine-containing neurons in Parkinson's disease.**

## Introduction

Nobel prize-winning work by Arvid Carlsson and colleagues in 1958 first demonstrated that dopamine is a neurotransmitter, rather than being only an intermediate step in the synthesis of norepinephrine (noradrenaline), and a neurotransmitter that plays an important role in regulating motor behavior. The first pharmacological evidence for the existence of specialized receptors for dopamine was obtained five years later, but it was not until 1972 that the identification of dopamine-stimulated adenylate cyclase, the enzyme that converts ATP into the "second messenger" cyclic AMP, made possible the biochemical characterization of a dopamine receptor. Later that decade, further pharmacological and biochemical chacterization of dopamine receptors, including their division into D1 and D2 subtypes, was made possible by the development of radioisotopically labeled ligands, or radioligands, that bind to dopamine receptors with high affinity and selectivity. What might be considered the modern era of dopamine receptor research began with the determination of the DNA sequence and predicted protein sequence of the D2 dopamine receptor and the subsequent identification of additional dopamine receptor subtypes.

## Dopamine Receptor Subtypes

Virtually all neurotransmitters and hormones activate multiple receptor subtypes—distinct classes of receptors that share relatively high affinity for a single neurotransmitter but that differ in structure, affinity for drugs, signaling, and distribution. It is common for the distribution of receptor subtypes to overlap, so that one organ or brain region may have multiple receptor subtypes for any neurotransmitter or hormone that is present.

### D1-Like and D2-Like Receptor Subfamilies

Prior to the molecular cloning of dopamine receptors, they were divided into D1 and D2 subtypes on the basis of several criteria. D1 and D2 receptors have distinct pharmacological profiles; in particular, D2 receptors have high affinity for benzamide and butyrophenone antagonists such as sulpiride and spiperone, respectively, while D1 receptors have high affinity for benzazepine antagonists such as SCH23390. D1 receptors stimulate adenylate cyclase and cyclic AMP accumulation, while D2 receptors inhibit the enzyme. Finally, the D1 and D2 receptors are physically distinct. For example, there are tissues such as the anterior and intermediate pituitary gland that have an abundance of D2 receptors but no D1 receptors. Furthermore, it is possible to cause selective damage to cell bodies in the substantia nigra or in the neostriatum of the rat brain, using lesions that spare axons and axon terminals, and with these lesions to cause a preferential reduction in the abundance of D2 receptors (substantia nigra) or D1 receptors (neostriatum), thus demonstrating the differential localization of the subtypes on cell bodies and axon terminals in these brain regions. The molecular cloning of additional subtypes of dopamine receptors revealed the existence of

 © 2004, Elsevier Inc. All Rights Reserved. 

two receptor subfamilies, in each of which the subtypes share D1-like or D2-like characteristics.

### Molecular Cloning of Dopamine Receptor Subtypes

Following the cloning of DNA encoding receptors determined to be identical to the pharmacologically characterized D1 and D2 receptors, several additional subtypes were identified in 1990–91. DNA sequence information was a new tool for classification of receptors, in that receptors belonging to the same subfamily are more similar in DNA and protein sequence. Thus, there are many amino acids that are common in D1 and D5 receptors (Figure 1A), but fewer that are shared among all the dopamine receptor subtypes (Figure 1B). D1 and D5 receptors are both D1-like receptors, whereas D2, D3, and D4 receptors belong to the D2-like receptor subfamily in terms of sequence identity and affinity for drugs.

## Structural Characteristics of Dopamine Receptors

Most hormone and neurotransmitter receptors are integral membrane proteins that span the cell membrane at least once, so that one portion of the receptor lies outside the cell and another portion lies inside. Dopamine receptors all belong to the superfamily of proteins called 7-transmembrane receptors, because they traverse the membrane seven times, serpentine receptors because of the manner in which the wind back and forth across the membrane, or G protein-coupled receptors because most of the effects of neurotransmitter binding to the receptors are mediated by activation of G proteins. The superfamily of 7-transmembrane receptors includes receptors for light, odors, and calcium, in addition to most neurotransmitters. Although all dopamine receptors have seven membrane-spanning regions with four intracellular domains (loops 1–3 and the carboxy terminus) and four extracellular domains (the amino terminal extension and extracellular loops 1–3), the D1-like receptors have relatively short third intracellular loops and relatively long carboxy termini compared to the D2-like receptors (Figure 1).

As illustrated in Figure 1, the presence of specific amino acid residues at certain locations across receptor subtypes, or amino acid sequence homology, is highest within the membrane-spanning regions of 7-transmembrane receptors. This is primarily because maintaining the three-dimensional structure of a receptor so that it is relatively quiescent until activated by the binding of a neurotransmitter such as dopamine requires precise packing of the membrane-spanning alpha-helices and a network of interhelical bonds that constrain the movement of the helices. Random mutations in these regions would be often deleterious and selected against because of the relatively high likelihood of disrupting helix packing or interhelical bonds. Within a family of receptors for one neurotransmitter or for several structurally related neurotransmitters such as the catecholamines dopamine and norepinephrine (noradrenaline), sequence homology also reflects the conservation of amino acids that bind the neurotransmitter; for small molecule neurotransmitters such as dopamine, these amino acids are typically in the membrane-spanning helices, which form a water-accessible binding pocket within the membrane lipid bilayer.

## Dopamine Receptor Subtype-Selective Drugs

D1 and D2 receptor-selective drugs have been very useful for differentiating between the behavioral and biochemical effects of D1-like or D2-like receptor stimulation. There are numerous commercially available potent and selective D2-like receptor agonists such as quinpirole and 7-OH DPAT, and antagonists such as spiperone and nemonapride (YM-09151-2). Although there are fewer D1-like receptor-selective drugs, the selective benzazepine antagonist SCH23390 and agonists such as SKF38393 and 6-chloro-APB (SKF82958) have been used to demonstrate that blockade or stimulation of D1-like receptors has significant behavioral consequences.

There is great interest in developing drugs that differentiate between D1 and D5 receptors or among the D2-like receptors, both as research tools and because of the possibility that selective blockade or stimulation of only one subtype will be a more effective treatment for a disease that is currently treated by blockade or stimulation of the D1-like or D2-like subfamily (Table I). It is still not possible to differentiate pharmacologically between D1 and D5 receptors, two subtypes with very similar sequence in the membrane-spanning helices (Figure 1A). On the other hand, the use of antagonists that are highly selective for only one of the three subtypes of the D2-like receptor subfamily, particularly for the D4 receptor, has demonstrated that dopamine binding to D3 or D4 receptors in rats has behavioral effects that are very different from D2 receptor-stimulated locomotor activation.

## Dopamine Receptor Signaling

Most of the effects of dopamine receptors on cellular function are mediated by activation of heterotrimeric

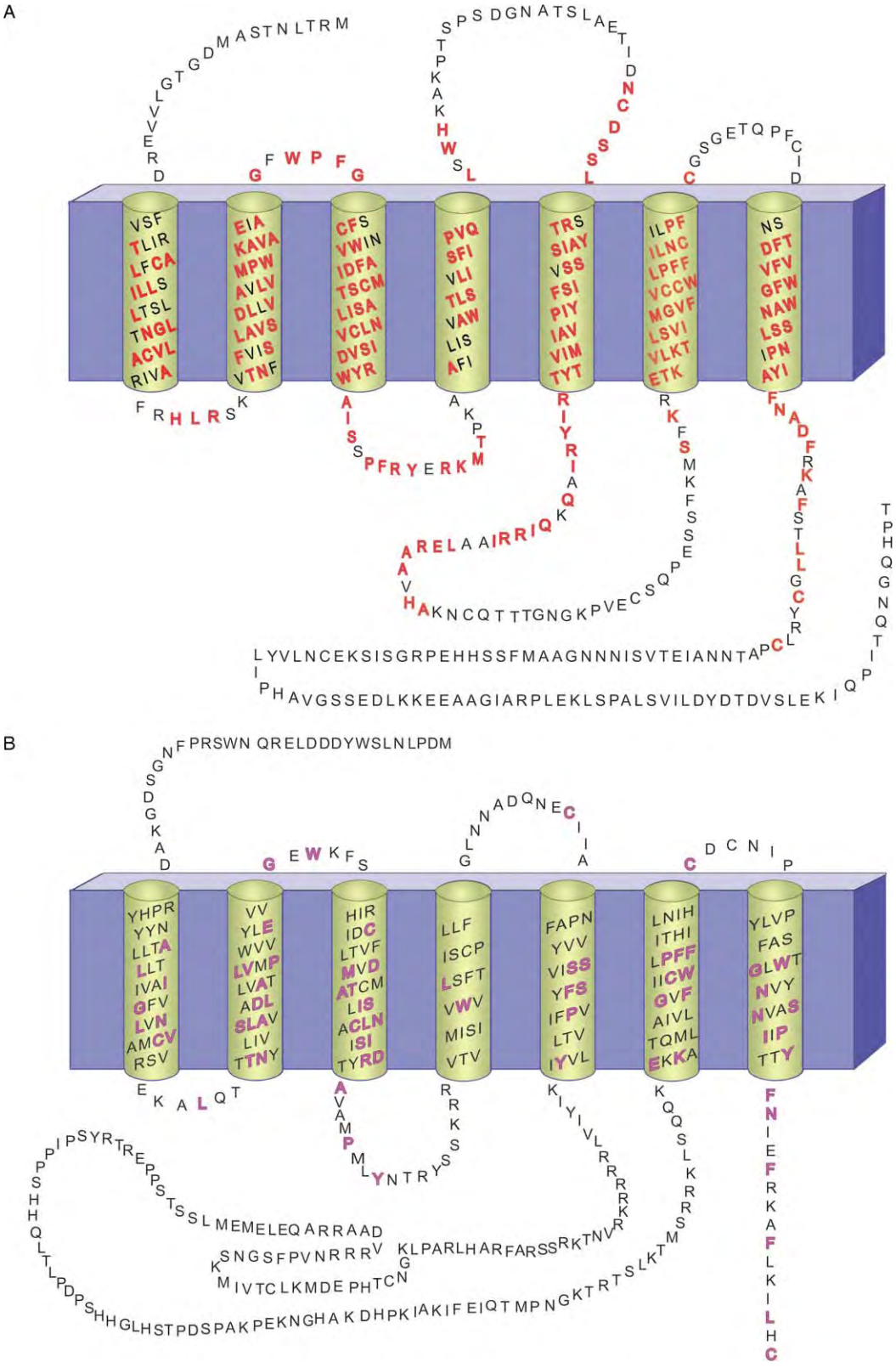



**FIGURE 1** Predicted amino acid sequence and membrane-spanning segments of the D1 (A) and D2 (B) dopamine receptor subtypes. The figure is oriented so that the extracellular regions of each receptor are above the shaded transmembrane helices and the intracellular regions are below. Amino acids colored red (A) are shared between D1 and D5 receptors, whereas amino acids colored purple (B) are shared among all of the dopamine receptor subtype.

**TABLE I**

**Features of Human Dopamine Receptor Subtypes**

| Type of feature | Dopamine receptor subtype | | | | |
|---|---|---|---|---|---|
| | **D1** | **D5** | **D2** | **D3** | **D4** |
| Length (amino acids) | 446 | 477 | 443[a] | 400 | 387[b] |
| Introns in coding region | No | No | Yes | Yes | Yes |
| G protein coupling | $G\alpha_s/G\alpha_{olf}$ | $G\alpha_s$ | $G\alpha_{i/o}$ | $G\alpha_{i/o}$ | $G\alpha_{i/o}$ |
| Agonists | SKF38393 | SKF38393 | Quinpirole | Quinpirole | Quinpirole |
| | 6-Cl-APB | 6-Cl-APB | 7-OH-DPAT | 7-OH-DPAT | 7-OH-DPAT |
| | | | | PD128907 | PD168077 |
| Antagonists | SCH23390 | SCH23390 | L-741,626 | GR 103691 | L-741,742 |
| | | | | U99194 | L-745,870 |
| Radioligands | [$^3$H]SCH23390 | [$^3$H]SCH23390 | [$^3$H]spiperone | [$^3$H]spiperone | [$^3$H]spiperone |
| | [$^{125}$I]SCH23982 | [$^{125}$I]SCH23982 | [$^3$H]YM-09151-2 | [$^3$H]YM-09151-2 | [$^3$H]YM-09151-2 |
| | | | | [$^3$H]7-OH-DPAT | |
| Localization[c] | Caudate-putamen, cerebral cortex, substantia nigra, parathyroid gland | Hippocampus, cerebral cortex | Caudate-putamen, substantia nigra, intermediate and anterior pituitary gland | Nucleus accumbens, olfactory tubercle | Cerebral cortex, hypothalamus, olfactory bulb |
| Splice variants | None | None | $D2_L/D2_S$ | D3nf[d] | None |
| Allelic variants[e] | None | Leu88 → Phe | Val96 → Ala | Ser9 → Gly | Gly11 → Arg |
| | | Ala269 → Val | Pro310 → Ser | | 12 bp repeat in exon 1 |
| | | Pro330 → Gln | Ser311 → Cys | | 21 bp deletion in exon 1 |
| | | Cys-335 → stop | | | 13 bp deletion in exon 1 |
| | | Asn351 → Asp | | | Val194 → Gly |
| | | Ser453 → Cys | | | 48 bp repeat in exon 3 |

[a] Length of $D2_L$; $D2_S$ is 414 amino acids long.

[b] Length of the variant with two repeats (D4.2) although actual length depends on the number of repeat units, which varies from 2 to 10.

[c] Selected tissues and brain regions with relatively high expression of the subtype are listed.

[d] D3nf is a frame-shifted variant with D3 receptor sequence only through the fifth membrane-spanning helix. Although shorter truncated splice variants of D3 mRNA also exist, D3nf is the only one for which expression of the protein has been demonstrated.

[e] Only variants that alter the protein sequence are listed.

GTP-binding proteins called G proteins. Binding of dopamine to amino acids within the membrane-spanning helices of a dopamine receptor disrupts the interhelical bonds that hold the receptor in an inactive state. The resulting movement of one or more of the helices exposes amino acids on the cytoplasmic face of the membranes that bind to and activate G proteins. D1-like and D2-like receptor have very different signaling properties as a result of differential selection of G protein subtypes.

### G Protein Coupling

Heterotrimeric G proteins are composed of $\alpha$-, $\beta$-, and $\gamma$-subunits, and are generally named according to the subtype of $G_\alpha$ subunit. D1-like receptors that have been stimulated by dopamine or other D1 receptor agonists activate two G proteins that stimulate adenylate cyclase, $G_{\alpha s}$ and the closely related G protein $G_{\alpha olf}$ (Table I). (The latter G protein was first identified as mediating olfactory responses to receptors for odorants.) D2-like receptors activate the $G_{\alpha i}$ subtypes, named after their ability to inhibit adenylate cyclase, and the closely related G protein $G_{\alpha o}$. That these G proteins are substrates for ADP-ribosylation by cholera toxin, which persistently activates $G_{\alpha s}$, or pertussis toxin, which inactivates $G_{\alpha i/o}$, has contributed importantly to determining their role in dopamine receptor signaling.

### Signaling Pathways

As suggested by their activation of G proteins that stimulate adenylate cyclase, most effects of D1-like receptor stimulation are mediated by increased levels of cyclic AMP and cyclic AMP-dependent protein phosphorylation. D1 receptor-stimulated protein phosphorylation alters the activity of other protein kinases, receptors, protein phosphatases, ion channels, and transcription factors. D2-like receptors inhibit the activity of adenylate cyclase via $G_{\alpha i}$. D2-like receptors also modulate many other signaling pathways, most of them as a result of the liberation of G protein $\beta\gamma$-subunits that occurs when 7-transmembrane receptors activate $G_{\alpha i/o}$ proteins. $G_{\beta\gamma}$-regulated pathways that are regulated by stimulation of D2-like receptors include several forms of adenylate cyclase, ion channels, phospholipase C, and mitogen-activated protein (MAP) kinases. Although the basic mechanism of activation of heterotrimeric G proteins by receptors is probably the same for all receptor-G protein combinations, for reasons that have not been determined, activation of other types of G proteins such as $G_{\alpha s}$ does not produce the same $G_{\beta\gamma}$-mediated signaling that is observed after receptor-mediated activation of $G_{\alpha i/o}$.

## Distribution of Dopamine Receptors

The D1 and D2 dopamine receptors are overall the most abundant dopamine receptor subtypes. The D1 receptor is expressed most highly in brain, with lower expression in periperheral tissues such as the parathyroid gland, renal, mesenteric, and coronary vascular beds, and the kidney. Within the brain, the D1 receptor is most abundant in the caudate-putamen and other basal forebrain nuclei such as the nucleus accumbens, and is also found in the cerebral cortex and the substantia nigra *pars reticulata*. The D5 receptor is expressed at much lower levels in the same basal forebrain nuclei, the cerebral cortex, and hippocampus. The D2 receptor is expressed most highly in the intermediate pituitary gland, with abundant expression also observed in the anterior pituitary and in a number of brain regions including the caudate-putamen and other nuclei of the basal ganglia, the substantia nigra *pars compacta*, and the cerebral cortex. The D4 receptor is expressed in most of the same forebrain regions as the D2 receptor and in the cerebral cortex, albeit at a lower level. Whereas the D2 receptor is most abundant in dorsal areas of the striatum, the D3 receptor is also expressed in the basal forebrain but is more abundant in the ventral nuclei (nucleus accumbens, olfactory tubercle).

## Dopamine Receptor Variants

Although there are only five mammalian dopamine receptor genes that give rise to D1–5 receptors, four of the genes produce multiple receptor variants (Table I). For example, the D2 receptor has two variants that result from alternative splicing of the RNA that is transcribed from the *DRD2* gene. The long form of the D2 receptor, $D2_L$, contains a 29-amino-acid insert encoded by an exon that is spliced out of the short form, $D2_S$. Recent evidence suggests that $D2_S$ and $D2_L$ might serve very different functions as autoreceptors that regulate dopamine release and as postsynaptic receptors that mediate the actions of dopamine on non-dopaminergic neurons, respectively. In contrast to splice variants that are derived from a single gene sequence and that are present in everybody, there are also dopamine receptor variants transcribed from distinct gene sequences, so that each individual expresses only one or two of the two or more existing variants. For example, there are at least 27 allelic variants of the human D4 receptor that have from 2 to 10 copies of an imperfectly repeated 48 nucleotide (16 amino acid) sequence in the third intracellular loop of the receptor. There is considerable interest in the possibility that these allelic variants also vary in functional characteristics.

## Therapeutic Uses for Dopamine Receptor Agonists and Antagonists

By far the most common therapeutic purpose for drugs that act on dopamine receptors is the use of D2 receptor antagonists for the treatment of schizophrenia and other psychoses. D2-like receptor antagonists are also used for the treatment of nausea and vomiting, for delerium or dementia of unknown cause, and for symptomatic treatment of hyperactive movement disorders such as Tourette's syndrome and Huntington's disease. Dopamine receptor agonists alleviate the symptoms of Parkinson's disease, and are often used in combination with the dopamine precursor L-DOPA. The D2-like receptor agonist bromocriptine is used to treat hyperprolactinemia because of the inhibitory effect of anterior pituitary D2 receptors on prolactin secretion. When hyperprolactinemia results from a prolactin-secreting tumor, treatment with bromocriptine also decreases the size of the tumor. Dopamine or D1 agonists such as fenoldopam cause D1 receptor-mediated vasodilation, thus increasing glomerular filtration, renal blood flow, and sodium excretion, and are used to manage types of shock associated with loss of cardiac output and compromised renal function.

## See Also the Following Articles

$G_i$ Family of Heterotrimeric G Proteins • $G_q$ Family • $G_s$ Family of Heterotrimeric G Proteins • $G_{12}/G_{13}$ Family • Neurotransmitter Transporters

## Glossary

**affinity** Term for how tightly a drug binds to a receptor, expressed as the concentration of drug that occupies half of the available receptors. High affinity means that a drug will bind at very low concentrations.

**agonist** A drug that binds to and activates a receptor. Dopamine is the endogenous agonist for dopamine receptors.

**antagonist** A drug that binds to a receptor without activating it, and thus prevents the binding of an agonist.

**receptor** A protein, usually associated with the cell membrane, that binds a neurotransmitter or hormone and initiates a biological response.

**second messenger** A chemical signal elicited by an activated receptor that transmits information within a cell. The extracellular neurotransmitter is the first messenger, and whereas some receptors transduce information about the neurotransmitter concentration into an electrical response, other receptors convert that information into altered abundance of an intracellular second messenger such as cyclic AMP.

## Further Reading

Bunzow, J. R. (1988). Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature* **336**, 783–787.

Emilien, G., Maloteaux, J. M., Geurts, M., Hoogenberg, K., and Cragg, S. (1999). Dopamine receptors – physiological understanding to therapeutic intervention potential. *Pharmacol. Ther.* **84**, 133–156.

Jose, P. A., Eisner, G. M., and Felder, R. A. (1998). Renal dopamine receptors in health and hypertension. *Pharmacol. Ther.* **80**, 149–182.

Meador-Woodruff, J. A., Damask, S. P., Wang, J., Haroutunian, V., Davis, K. L., and Watson, S. J. (1996). Dopamine receptor mRNA expression in human striatum and neocortex. *Neuropsychopharmacology* **15**, 17–29.

Neve, K. A., and Neve, R. L. (eds.) (1997). *The Dopamine Receptors.* Humana Press, Totowa, NJ.

Sealfon, S. C., and Olanow, C. W. (2000). Dopamine receptors: From structure to behavior. *Trends Neurosci.* **23**, S34–S40.

Sidhu, A., Laruelle, M., and Vernier, P. (eds.) (2003). *Dopamine Receptors and Transporters: Function, Imaging, and Clinical Implication.* Marcel Dekker, New York.

Vallone, D., Picetti, R., and Borrelli, E. (2000). Structure and function of dopamine receptors. *Neurosci. Biobehav. Rev.* **24**, 125–132.

## Biography

Kim A. Neve is a Senior Research Career Scientist at the Portland Veterans Affairs Medical Center and a Professor in the Department of Behavioral Neuroscience at Oregon Health & Science University in Portland, Oregon. He holds a Ph.D. from the University of California, Irvine and received his postdoctoral training at the University of Pennsylvania. His principal research interest concerns the structure and function of dopamine receptors, and he has worked extensively on determining how the responsiveness of the receptors is regulated by dopamine.

# Dynactin

**Trina A. Schroer**
*The Johns Hopkins University, Baltimore, Maryland, USA*

**Dynactin is a multi-subunit protein complex that links many components of cells to microtubules and microtubule-based motors. In addition to serving as an adapter that tethers and/or allows movement of cellular structures, dynactin also enhances the activity of some motors by increasing their processivity.**

## Overview of Dynactin Composition and Structure

Dynactin contains a total of 24 individual polypeptide subunits that are encoded by 11 different genes (Figure 1). The locations and nearest neighbors of most dynactin subunits have been identified using antibody labeling and biochemical analysis (Figure 2). Most of dynactin's mass is contained in a rod-like structure that is an octameric polymer of the actin-related protein, Arp1. The subunits p62, Arp11, p27, and p25 are organized into a disk-shaped complex that sits at one end of this polymer. The actin-capping protein (CapZ) $\alpha/\beta$ heterodimer is present at the other end of the Arp1 polymer. Dynactin also contains a single monomer of conventional cytoplasmic $\beta$-actin, the precise location of which has not been rigorously defined. The entire rod-like domain is thought to mediate interactions of dynactin with a wide variety of cellular structures.

The other prominent structural domain of dynactin is a flexible shoulder and arm complex that projects away from the Arp1 rod. This complex contains the largest dynactin subunit, $p150^{Glued}$, dynamitin (p50), and p24 in a stoichiometric ratio of 2:4:2. The projecting domain is where dynactin binds motors and microtubules.

Dynactin structure has been imaged in the electron microscope (EM) by a variety of methods, including negative-staining EM and scanning transmission EM. The most detailed information has been derived from platinum replicas of rotary shadowed, quick-frozen, deep-etched molecules. The dynactin structure obtained in this manner is the basis of the cartoon shown in Figure 2. Till the time of preparation of this entry, no high resolution structural information about dynactin as a whole or any of its individual subunits (aside from actin and CapZ) was available.

## Dynactin Functions

### Functions Associated with Dynein-Based Motility

Dynactin was first discovered as a large (20S) protein complex that copurified with the minus end-directed motor, cytoplasmic dynein. Dynactin was found to stimulate dynein's ability to translocate membrane vesicles *in vitro*. The name dynactin derives from its *dyn*ein-*act*ivating activity.

#### Adapter Function

An important function of dynactin is to serve as an "adapter" that allows cytoplasmic dynein to bind a variety of cargoes. This adapter function relies on dynactin's distinct dynein and cargo-binding domains. Dynein is the predominant minus end-directed motor activity in most cells and therefore virtually any subcellular structure or macromolecular complex that moves toward the cell center is translocated by dynein, usually in conjunction with dynactin. Dynein cargoes include membranous organelles such as the Golgi complex, the ER–Golgi intermediate compartment (ERGIC), endosomes, lysosomes, and mitochondria. Dynein can also move lipid droplets, aggresomes, viral capsids, and centrosome components toward the cell center.

Dynactin also contributes to the binding of dynein to large cellular structures such as centrosomes, mitotic spindle poles, chromosomes, the nucleus, and the plasma membrane. Dynein associates with centrosomes beginning in S-phase and remains at spindle poles during mitosis. Centrosomal dynactin is required for dynein binding. The persistent action of dynein at spindle poles is required for the retention and organization of microtubules. Dynactin is also required for proper binding of dynein to chromosome kinetochores, which is important for capture of spindle microtubules in prometaphase. Dynein may also power the lateral sliding of chromosomes along microtubules and contribute to attachment to microtubules that must undergo

 © 2004, Elsevier Inc. All Rights Reserved. 

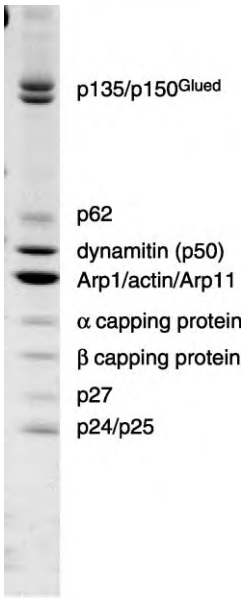



**FIGURE 1** Dynactin composition. Dynactin's 11 distinct polypeptide subunits are shown on this Coomassie blue-stained, SDS polyacrylamide gel.

disassembly at their plus ends during prometaphase and anaphase. Just prior to mitosis, dynactin and dynein are recruited to the outer nuclear membrane. Dynein-driven movement of the nuclear envelope along microtubules pulls the entire centrosomal microtubule array toward the nucleus. This causes centrosomes to become closely associated with the nuclear surface and contributes to nuclear envelope rupture.

Dynein binds to the cell cortex via dynactin. As in the case of dynein bound to the nuclear envelope, the force exerted by cortical dynein "reels in" microtubules, pulling with them any structures to which they might be attached. This process can reorient the entire microtubule array and contributes to the long-range movements of nuclei that occur during mitosis and cellular morphogenesis.

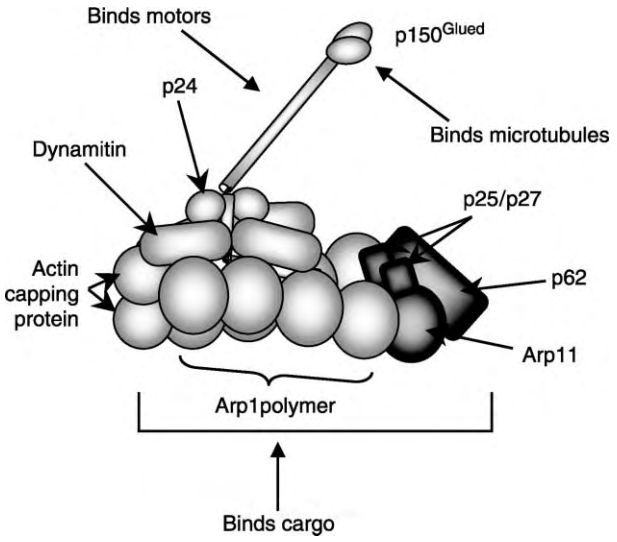



**FIGURE 2** Subunit organization in dynactin. The three different binding domains are indicated. The precise location of conventional actin is unknown.

### Processivity Enhancement

In the absence of dynactin, cytoplasmic dynein's two motor domains are able to travel only a very short distance along the microtubule lattice. Dynactin's $p150^{Glued}$ subunit provides a second set of microtubule binding sites that holds dynein in the vicinity of the microtubule. This low affinity binding allows dynein to move over longer distances, making it a more processive motor.

### Interactions with other Motors

Dynactin may bind motors of the kinesin family to target them to cargoes and enhance processivity. Evidence exists suggesting that $p150^{Glued}$ binds Eg5/BimC, a plus end-directed motor that is localized at spindle poles where it contributes to microtubule organization. Membranous organelles that undergo dynamic bidirectional movements, such as late endosomes and pigment granules, must also be able to bind multiple motors. Dynactin on the surface of such membranes may be able to recruit both dynein and the heterotrimeric, plus end-directed motor, kinesin II (KIF3), thereby allowing bidirectional translocation.

## Dynein-Independent Functions

Dynactin can support non-motile interactions of microtubules with cellular components in a manner that is independent of motor binding. This cross-linking function depends on dynactin's distinct microtubule and cargo-binding domains. Dynactin associates with microtubules at their plus ends, their minus ends, and along their length. At plus ends, dynactin may help control the rate of microtubule growth and shrinkage, perhaps by recruiting or working with other plus end-binding factors. Dynactin is also seen at sites where microtubule minus ends converge, such as centrosomes, at times when dynein is not. Centrosomal dynactin is required for microtubule anchoring at this site. Dynactin may interact with microtubule minus ends directly, or it might recruit other minus end-binding factors. Subunits of dynactin's cargo-binding domain are able to associate with a wide array of intracellular molecules and macromolecular complexes that are still being identified. This may allow dynactin to bind cellular components to microtubules transiently. This would prevent them from diffusing freely in cytosol and could cause them to be retained at particular sites within the cell, for example, in the cytoplasm rather than the nucleus.

## Structure and Function of Individual Dynactin Subunits

### Structure

Dynactin subunits can be grouped into four structural families: (1) proteins whose structures resemble actin (actin itself, plus the actin-related proteins Arp1 and Arp11) or actin-binding proteins (CapZ), (2) $\alpha$-helical, elongated proteins (p150$^{Glued}$, dynamitin and p24), (3) $\beta$-helix proteins (p25 and p27), and (4) Cys/His RING/LIM domain proteins (p62).

#### Actin-Related or Actin-Binding Proteins

Dynactin contains three proteins of the actin superfamily: $\beta$-(cytoplasmic) actin, Arp1, and Arp11. Arp1 is quite similar (~65% identical) to conventional actin and is the only Arp that has been shown to assemble into filaments in vitro and hydrolyze ATP. Arp11 is less similar to actin but can copolymerize with actin and bind Arp1 *in vitro*. Sequence comparisons suggest that Arp11 is most divergent at its pointed end face. Arp11 has been localized to the pointed end of the Arp1 filament where it may bind Arp1 directly. The CapZ species found in dynactin contains the $\beta_2$ isoform only. Among the dynactin subunits, these five dynactin polypeptides are the most highly conserved between species.

#### $\alpha$-Helical Proteins

The polypeptides p150$^{Glued}$, dynamitin and p24 are the least highly conserved of all the dynactin subunits, suggesting that their overall structures may play a more important role in dynactin function than their specific sequences. p150$^{Glued}$ forms a parallel homodimer that is stabilized by two long coiled coils, one ~300 amino acids long and the other ~125 amino acids long (Figure 3). The 200 amino acid N-terminal domains comprise the globular heads found at the tip of the projecting arm. Only the N-terminal ~400 amino acids are exposed in the projecting arm, leaving the C-terminal two-thirds to associate with other dynactin subunits in the elastic shoulder that is associated with the Arp1 filament. The structural features of the central region and C terminus of p150$^{Glued}$ have not yet been defined. The p150$^{Glued}$ mRNA is spliced at multiple sites that are still being characterized. This may yield p150$^{Glued}$ protein isoforms that exhibit differential binding to microtubules and other dynactin subunits.

Isolated dynamitin is an elongated tetramer, but it folds into a more compact structure when associated with other dynactin subunits. Dynamitin and p24 (2:1 stoichiometric ratio) associate stably with each other to form a complex that plays a critical role in dynactin integrity. Free dynamitin is able to displace endogenous dynamitin, p24 and p150 from the Arp1 filament. The location of dynamitin within the dynactin molecule has not been rigorously determined, but its known neighbors and general structural properties suggest that it is a major structural component of the shoulder.

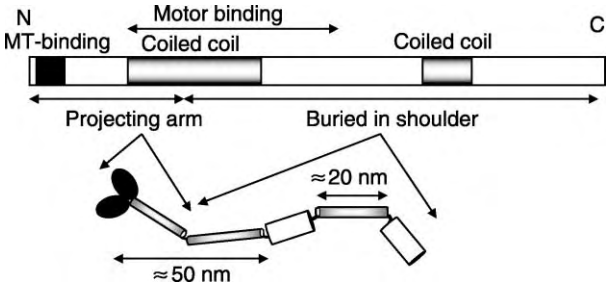



**FIGURE 3** Two representations of the domain organization of p150$^{Glued}$. Top: A schematic of the primary sequence with predicted structural features and sites of known interactions indicated. Bottom: A cartoon illustrating the N-terminal globular heads and location and approximate lengths of the two coiled-coil domains. The structural features of the open boxed regions have not been determined.

#### $\beta$-Helix Proteins: p25 and p27

Dynactin contains one copy each of p27, p25, Arp11, and p62, which are closely associated in with each other. p27 and p25 are evolutionarily related and contain a polypeptide repeat motif that is characteristic of $\beta$-helical proteins. $\beta$-helix proteins are common in proteins from plants and pathogenic organisms, but rarer in animal cells. Most $\beta$-helices are elongated and possess an extended binding face or groove. The $\beta$-helices in p25 and p27 are predicted to be short, but the two proteins may associate with each other end-to-end to provide a longer-binding face. The unusually alkaline pI of the p25/p27 dimer suggests this part of dynactin may associate with acidic binding partners.

#### RING/LIM Domain Protein: p62

Very little is known about p62 structure, but it has been shown to associate with actin, Arp11, and Arp1. p62 contains a Cys/His-rich motif of the RING/LIM type that may chelate a metal ion and contribute to interactions with other dynactin subunits and/or other proteins. p62 contains a single consensus site for phosphorylation by cell cycle kinases (Cdks) that may govern its cell cycle-dependent binding to nuclei.

### Binding Activities

Dynactin can interact with a wide variety of different cellular components. Its binding partners fall into three

classes: (1) microtubules, (2) motors, and (3) motile cargoes or non-motile subcellular docking sites. Each type of interaction is supported by a different part of the dynactin molecule (Figure 2).

### Microtubule Binding

Microtubule binding is mediated by a CAP-Gly (i.e., cytoskeleton-associated protein that is glycine-rich) motif found in the N-terminal "head" domain of each $p150^{Glued}$ subunit (Figure 3). CAP-Gly motifs have been found in other microtubule binding proteins such as CLIP-170, Bik1 and the tubulin-folding cofactors, Factor B and E. The affinity of microtubule binding is regulated by phosphorylation, possibly at multiple sites. The ability of dynactin to bind microtubules with a range of affinities may allow it to work as either a processivity factor or a tethering molecule.

### Motor Binding

$p150^{Glued}$ also contributes to motor binding. Its best-characterized binding partner is cytoplasmic dynein, but $p150^{Glued}$ has also been reported to interact with the kinesin family motors, Eg5 and kinesin II. Motors bind to the central portion of $p150^{Glued}$ (amino acids ~200–800; Figure 3). It is not known whether this element contains a single-motor-binding site or whether distinct sites bind different motors. In addition to $p150^{Glued}$, nearby dynactin subunits such as dynamitin and p24 may contribute to binding to cytoplasmic dynein and other motors.

### Binding to other Cellular Components

*Membranes* Dynactin localizes to many cellular membranes, including the Golgi complex, ERGIC, endocytic pathway components, the nuclear envelope and the plasma membrane. All these interactions are thought to involve dynactin's Arp1 filament domain. Arp1 can associate with the actin-binding region of at least one spectrin isoform, which might provide a general mechanism for binding to the Golgi complex, nuclear envelope, and plasma membrane. Nuclear envelope binding also utilizes p62 and Arp11.

*Other Cytoplasmic Structures* The association of dynactin with lipid droplets, intracellular pathogens and cytoplasmic signaling complexes also appears to involve the Arp1 filament domain. The mechanisms for binding to most of these structures have not yet been defined. Cytoplasmic signaling complexes appear to bind dynactin via p27 and p25. These subunits are loosely associated with dynactin and may provide a mechanism for promoting transient and/or regulated interactions with a variety of cellular components.

*Chromosomes* Dynactin is bound to chromosome kinetochores during mitosis. As in the case of other structures that bind dynactin, kinetochore binding probably involves multiple dynactin subunits. One known contributor to this interaction is dynamitin, which binds the kinetochore component, ZW10, directly.

## See Also the Following Articles

Actin-Related Proteins • Centrosomes and Microtubule Nucleation • Dynein • Microtubule-Associated Proteins

## Glossary

**centrosome** A complex organelle located at the center of many cells that is the major site of microtubule nucleation and in some cells, focusing of minus ends.

**dynein** A massive, multisubunit mechanoenzyme that powers movement toward the minus ends of microtubules.

**microtubule** A 25 nm diameter hollow cytoskeletal polymer comprising 11–15 parallel protofilament strands of $\alpha/\beta$ tubulin heterodimers.

**motor** An ATPase that performs mechanical work as part of its ATP hydrolytic cycle.

**processivity** The ability of an enzyme to catalyze a reaction on multiple, successive subunits of a polymeric substrate.

## Further Reading

Allan, V. (2000). Dynactin (Quick guide). *Curr. Biol.* **10**, R432.

Holleran, E. A., Karki, S., and Holzbaur, E. L. (1998). The role of the dynactin complex in intracellular motility. *Int. Rev. Cytol.* **182**, 69–109.

Schafer, D. A., and Schroer, T. A. (1999). Actin-related proteins. *Annu. Rev. Cell Dev. Biol.* **15**, 341–363.

Vale, R. D. (2003). The molecular motor toolbox for intracellular transport. *Cell* **112**, 467–480.

## Biography

Dr. Trina A. Schroer is a Professor in the Department of Biology at The Johns Hopkins University in Baltimore, Maryland. Her principal research interests are cytoarchitecture and subcellular motility, with a particular focus on the functions of the microtubule cytoskeleton and microtubule-based motors. She holds a Ph.D. from the University of California, San Francisco and received postdoctoral training at the Washington University School of Medicine. Dr. Schroer discovered dynactin as a postdoctoral fellow and has made significant contributions toward determining its composition, structure, and functions.

# Dynein

**K. Kevin Pfister**
*University of Virginia School of Medicine, Charlottesville, Virginia, USA*

**Dyneins are motor proteins that move along microtubules toward their minus ends. They are members of the AAA family of ATPases. Dyneins were first identified by Gibbons as the ATPases that are responsible for the propulsive flagella bending and he named them after the unit of force, the dyne. Many members of the dynein family have been found in organisms from yeast to humans. All dyneins are large protein complexes composed of one or more ~530 kDa polypeptides, known as heavy chains, and various accessory subunits from 10 to 150 kDa.**

## Characteristics of Dynein Complexes

Dyneins are found in many eukaryotes, including, fungi, worms, insects, and vertebrates, but analysis of *Arabidopsis* genome indicates that they are not found in plants. Dyneins are classified as either cytoplasmic or flagellar, and the flagellar dyneins are further divided into the outer arm and inner arm dyneins. Cytoplasmic dynein moves membranous organelles, kinetochores, and viruses along microtubules and assists in the assembly and function of the mitotic spindle. Cytoplasmic dynein is also responsible for retrograde axonal transport. The flagellar dyneins, also called axonemal dyneins, are the arms that project from the doublet axonemal microtubules of flagella and cilia. The flagellar dyneins generate the sliding force between outer doublet microtubules that is converted by other axonemal structures into the bending of cilia and flagella. The outer arms specify the flagellar beat frequency and the inner arms specify flagellar waveform. There is also a dynein responsible for the transport of protein complexes from the tip of cilia and flagella to their base that occurs between the axonemal microtubules and the flagellar membrane, known as intraflagellar transport.

Fungi, *Drosophila,*and mammals have provided the major sources and systems for the study of cytoplasmic dynein. The alga *Chlamydomonas*, which has two flagella, has been a useful model system for the study of flagellar dyneins. As many as ten different dynein complexes are found in animals and most of these are components of the flagella. Molecular genetic analyses indicate that genomes contain more than ten flagellar dynein heavy chain genes, one cytoplasmic dynein heavy chain gene, and the intraflagellar transport dynein heavy chain, which is most related to the cytoplasmic dynein heavy chain. Dynein complexes are composed of one, two, or three heavy chains, and each complex has various smaller accessory subunits (Tables I and II). Cytoplasmic dynein has two identical heavy chains. Flagellar outer arm dyneins have two or three different heavy chains depending on the species. Each flagella has many different inner arm dyneins. Inner arm dynein 1 has two different heavy chains, and there are at least six different inner arm dyneins each with one unique heavy chain.

The different dynein complexes hydrolyze MgATP at different rates, for example, the microtubule stimulated ATPase activity of cow brain cytoplasmic dynein is 0.3 $\mu$mol min$^{-1}$ mg$^{-1}$ and the activity of flagellar outer arm dynein is 2.0 $\mu$mol min$^{-1}$ mg$^{-1}$. Different dynein complexes also move along microtubules at different rates. An *in vitro* motility assay is used to quantify the velocity of dynein-based movement by using computer-enhanced video microscopy. Dynein is attached to a glass coverslip and when microtubules are added the dynein motor domains bind the microtubules. In the presence of MgATP, the dynein moves microtubules across the coverslip with the microtubule-plus ends leading. Cytoplasmic dynein from bovine brain moves microtubules at the rate of ~1 $\mu$s$^{-1}$ while sea urchin outer arm dynein moves microtubules at the rate of 3.5 $\mu$s$^{-1}$.

## The Dynein Heavy Chain

The central component of the dynein complex is the heavy chain, an ~530 kDa polypeptide of that contains the ATP-binding and hydrolysis site and a microtubule-binding domain. Often the heavy chain alone is referred to as a dynein. The heavy chain is an AAA ATPase. The C-terminal two-thirds of the heavy chain, ~350 kDa, is necessary for ATP-dependent microtubule binding. This domain contains six sequential AAA domains, with

 © 2004, Elsevier Inc. All Rights Reserved. 

**TABLE I**

**Composition of Cytoplasmic Dynein Complexes**

| | Cytoplasmic dynein | Intraflagellar transport dynein |
|---|---|---|
| Heavy chain | HC1a | HC1b |
| Number of heavy chains | 2 | nd (1?) |
| Intermediate chain[a] | IC74-1<br>IC74-2 | n |
| Light intermediate chain[a] | LIC1<br>LIC2 | LIC3 |
| Light chain[b] | Tctex1 family<br>Roadblock family<br>LC8 family | n |

n, none identified; nd, not determined.

[a] It is not known if the intermediate chains associate in the dynein complex as homodimers or heterodimers. The light intermediate chains associate in the dynein complex only as homoligomers.

[b] Each light chain family has at least 2 members.

~350 amino acid microtubule-binding region inserted between AAA domains 4 and 5. Consensus nucleotide-binding sites known as P loops are components of AAA domains 1 through 4, while AAA domains 5 and 6 lack consensus P loop motifs. The first P loop, P1, is the site of ATP binding and hydrolysis which generates force. In the presence of vanadate and inorganic phosphate, the heavy chains are cleaved by photolysis with ultraviolet light into two fragments at P1. The role of the other P loops is unknown although at least one of them binds nucleotide.

Structural analysis of the heavy chain indicates that the C-terminal motor portion forms a large globular domain known as the head (Figure 1). The head is made up of six or seven lobes arranged in a ring surrounding a central cavity. The individual AAA domains make six of the lobes. The microtubule binding region, two anti-parallel coiled coils separated by a globular region, projects from the head as a stalk. The N-terminal portion of the heavy chain extends from the head as a thin flexible stem across the head from the microtubule binding stalk. Two or three dynein heavy chains are often connected by their stems to form a common base. Most of the other subunits of the dynein complexes bind to the N terminus of the heavy chain at the base and they contribute to the motor protein's cargo-binding domain.

## Cytoplasmic Dynein

The mammalian cytoplasmic dynein (Figure 1 and Table I) has a native molecular weight of $1.5 \times 10^3$ kDa. It is a homodimer of two identical heavy chains associated with two ~74 kDa intermediate chains, two to four ~55 kDa light intermediate chains (LIC) and three pairs of light chains, the Tctex1, roadblock, and LC8 families. The heavy chain of this dynein is encoded by a single gene, but two genes have been identified for the mammalian intermediate and light intermediate chains and for each of the three light chain families. Intermediate chain and light intermediate chain isoforms are also generated by mRNA alternative

**TABLE II**

**Composition of Chlamydomonas Flagellar Dynein Complexes**

| | Outer arm | Inner arm 1 | Inner arm (a, c, d) | Inner arm (b, e, g) |
|---|---|---|---|---|
| Heavy chains | $\alpha$, $\beta$, and $\gamma$ | I$\alpha$ and I$\beta$ | a, c, or d | b, e, or g |
| Number of heavy chains | 3 | 2 | 1 | 1 |
| Intermediate chain | IC1<br>IC2 | IC140<br>IC138<br>IC110 | None | None |
| Light intermediate chain | None | None | None | None |
| Light chain | LC1/leucine rich<br>LC2/tctex2<br>LC3/Thioredoxin<br>LC4/Ca + 2 binding<br>LC5/Thioredoxin<br>LC6/LC8 homolog<br>LC7/roadblock homolog<br>LC8 | p22 (nk)<br>Tctex1<br>LC8 | Actin<br>p28 | Actin<br>Centrin<br>Novel actin<br>Related protein |

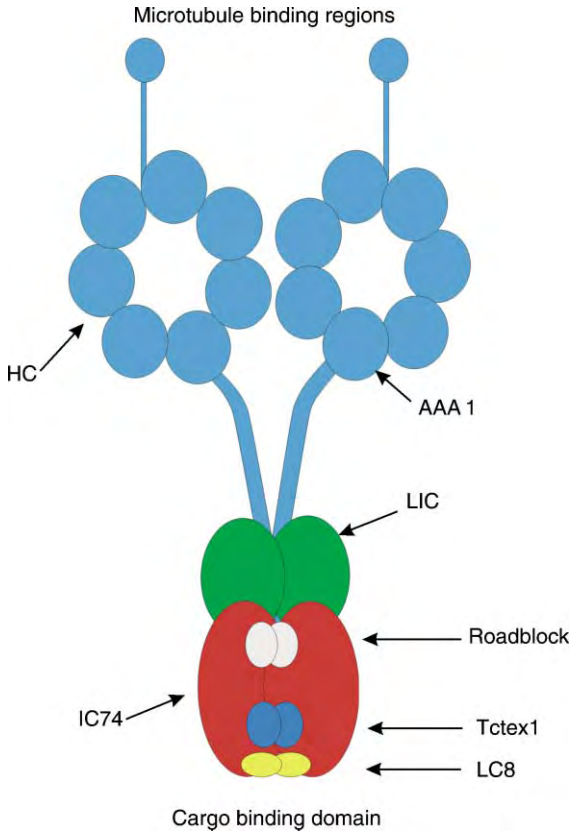



**FIGURE 1** Model for the structure of cytoplasmic dynein. The two heavy chains, in light blue, have heads with six to seven lobes. A microtubule binding stalk projects from each head, and the N-terminal stem binds the light intermediate chains (LIC) and the intermediate chains (IC74). The three light chains bind the intermediate chain. The smaller subunits define the cargo-binding domain.

splicing. The heavy chain dimerization domain is a large portion of its N terminus. Also in this region are the overlapping binding sites for the light intermediate chains and the intermediate chains.

The intermediate chains play an important scaffold role in the structure of the cytoplasmic dynein complex. In addition to binding the heavy chains, the intermediate chain binds the three light chain families. It also binds other proteins including the p150 subunit of dynactin, a protein that links dynein to its cargo. The intermediate chain has seven WD domains at its C terminus, which are necessary for binding to the heavy chain. The intermediate chain N terminus is a region predicted to form a coiled coil followed by the alternative splicing regions. The binding site for the dynactin subunit, p150 contains the N-terminal coiled coil region and the next ~60 amino acids. The Tctex1 and LC8 light chains bind the intermediate chain N terminus near to the alternative splicing region, while the Roadblock light chain binds near the first of the WD domains.

Cytoplasmic dyneins have many functions in cells, including transporting many organelles. They are important for maintaining Golgi position near the centrosome, for endosome movement, and for nuclear migration. During mitosis, they assist in the assembly of spindle poles, are used by kinetochore to move along microtubules, and generate pulling forces from the cell cortex to separate the spindle poles. In axons, cytoplasmic dynein is essential for retrograde membrane-bounded organelle transport in fast transport and for the movement of microtubules and neurofilaments. Several viruses including Adenovirus, Herpes, and HIV also utilize cytoplasmic dynein to move along microtubules during the infection process. The cytoplasmic dynein cargo-binding domain is made up of the intermediate chains, light intermediate chains, and the three light chain families. The best-characterized protein responsible for linking dynein to cargo is the dynactin complex, it is important for dynein binding to kinetochores and most membrane-bounded organelles. Dynactin also increases the processivity of cytoplasmic dynein, the distance dynein travels along a microtubule before detaching. Cytoplasmic dynein binds liposomes, artificial lipid membranes, so it may bind directly to some membranes. Cytoplasmic dynein also transports individual proteins, which bind directly to the intermediate chains, light intermediate chains, or the light chains.

The cytoplasmic dynein heavy chain, light intermediate chain, and intermediate chain subunits are phosphorylated *in vivo*. Light intermediate chain phosphorylation is correlated with cell-cycle regulation of dynein binding to membrane-bounded organelles. Intermediate chain phosphorylation regulates dynein binding to the p150 subunit of dynactin.

In yeast deletion mutations of dynein subunits are not lethal, but mitosis is disrupted. Deletion of the cytoplasmic dynein heavy chain is lethal in multicellular organisms such as mouse and *Drosophila*. Non-lethal mutations of cytoplasmic dynein heavy and light chains lead to axonal pathfinding defects and other pleotrophic developmental defects in *Drosophila*, and mutations in the mouse heavy chain result in age-dependent progressive degeneration of motor neurons. Cytoplasmic dynein also binds to and is modulated by the lissencephaly disease protein, Lis1. Lissencephaly is characterized by defects in the organization of the cortical regions of the mammalian brain.

## Flagellar Dyneins

Dyneins are the motor proteins that drive the bending of cilia and flagella. The flagellar dyneins are attached to the outer doublet microtubules of axonemes and generate the sliding force between neighboring outer doublet microtubules that is the basis for the propulsive bending of flagella that move cells through fluids.

The base of a flagellar dynein binds to one of the axonemal outer doublet microtubules, the A microtubule. The dynein head binds to the B microtubule of the neighboring outer doublet in an ATP-sensitive manner and utilizes the hydrolysis of ATP to push the B microtubule toward the tip of the axoneme. The arms are arranged in two rows along the microtubules, the outer and inner arms, and the dyneins in each row are different and have different functions. The outer arms specify the flagellar beat frequency and the inner arms specify waveform. While *Chlamydomonas* has fourteen flagellar dynein heavy chain genes, eight different flagellar dynein complexes have been purified from their flagella to date. There is one outer arm dynein complex and at least seven different inner arm complexes (Table II).

### Outer Arm Dynein

The *Chlamydomonas* outer arm dynein is a heterotrimer of three different heavy chains, however in some species, such as sea urchin, the outer arm is a heterodimer. The outer arm dynein also has two different intermediate chains, and eight light chains. Outer arm dynein binds to the microtubule through the outer arm docking complex. The outer arm dynein light chains are well characterized. LC1 is a member of a leucine-rich repeat family, and unlike the other light chains, it binds to the motor domain of the gamma heavy chain, not to its N terminus. LC3 and LC5, are thioredoxin homologues. LC4 is involved in the regulation of dynein by $Ca^{+2}$. LC6 and LC8 are members of the LC8 family. There is also a roadblock light chain, LC7, and LC2 is Tctex2. Although they bind to different cargo, the intermediate chain and light chain subunits, which make up the cargo-binding domains of cytoplasmic dynein and outer arm dynein are closely related. The C termini of the intermediate chains, the region, which interacts with the heavy chains, are conserved between outer arm and cytoplasmic dynein complexes. However, the N termini of outer arm and cytoplasmic dynein are different and are thought to allow the binding to the different cargo. Both cytoplasmic dynein and outer arm dynein have light chains that are members of the Tctex family. The two complexes also have roadblock light chains and share identical LC8 light chains. LC8 is also a component of the two-headed inner arm dynein, and is thus associated with all dynein complexes that have more than one heavy chain.

### Inner Arm Dynein

The inner arm dyneins are very heterogeneous (Table II). Inner arm dynein 1 has two different heavy chains, three different intermediate chains, and three light chains, including Tctex1 and LC8, both of which are also components of cytoplasmic dynein. The other six inner arm dyneins are organized into two groups. Each of the six dyneins has a single unique heavy chain and the heavy chains associate with two different sets of light chains. Three inner arm dynein heavy chains associate with actin and p28. The other three heavy chains associate with actin, an actin-related protein, and centrin. The functional significance the association of actin with these dyneins is unknown.

To convert from dynein-driven microtubule sliding to flagellar bending, the flagellar dyneins must be regulated. During flagellar bending, only a subset of the flagellar dyneins function at a time. The phosphorylation state of the inner arm dynein intermediate chain IC138 modulates this regulation. Genetic analysis has also identified several proteins that make up a dynein-regulatory complex, though the mechanism of the complex remains unknown.

## Intraflagellar Transport Dynein

The intraflagellar transport dynein is encoded by a single unique heavy chain. To date a unique light intermediate chain is the only identified accessory subunit which binds to this heavy chain. This dynein moves protein complexes from the flagellar tip to the base in the space between the outer double microtubules and the flagellar membrane. It is most closely related to the cytoplasmic dynein heavy chain.

### See Also the Following Articles

Actin Assembly/Disassembly • Actin-Capping and -Severing Proteins • Dynactin • Mitosis

### Glossary

**AAA ATPase** Super family of ATPases associated with various cellular activities found in all kingdoms of living organisms.

**axoneme** Cytoskeletal structure of the cilia and flagella composed of nine outer doublet microtubules with dynein arms and radial spokes, and two central microtubules with central pair projections.

**head** Globular C-terminal portion of the dynein heavy chain containing six AAA domains and the microtubule-binding stalk. It is the smallest portion of the heavy chain that has ATP-dependent microtubule binding.

**microtubule** A cytoskeletal polymer of tubulin dimers in ~13 protofilaments arranged as a hollow tube with two distinct ends, the plus and minus ends.

**stem** N-terminal of the dynein heavy chain, binds most other subunits of the dynein complex and can participate in dimerization with one or more other heavy chains.

## Further Reading

DiBella, L. B., and King, S. M. (2001). Dynein motors of the chlamydomonas flagellum. *Intl. Rev. Cytol.* **210**, 227–268.

Gibbons, I. R. (1988). Dynein ATPases as microtubule motors. *J. Biol. Chem.* **263**, 15837–15840.

Porter, M. E., and Sale, W. S. (2000). The 9 + 2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility. *J. Cell Biol.* **27**, F37–F42.

Vale, R. D. (2003). The molecular motor toolbox for intracellular transport. *Cell* **112**, 467–480.

Vallee, R. B., Shpetner, H. S., and Paschal, B. M. (1989). The role of dynein in retrograde axonal transport. *Trends Neurosci.* **12**, 66–70.

## Biography

K. Kevin Pfister is an Associate Professor of Cell Biology at the University of Virginia School of Medicine. His current major research interest is the function of cytoplasmic dynein in axonal transport. His lab characterized the cytoplasmic dynein intermediate chains, and with Dr. S. King identified the cytoplasmic dynein light chains. He earned a Ph.D. from Princeton University and served as a postdoctoral fellow at the University of Texas Southwestern Medical Center.

# Editors-in-Chief


## William J. Lennarz

State University of New York at Stony Brook, Stony Brook, New York, USA

***Section: Lipids, Carbohydrates, Membranes and Membrane Proteins***


WILLIAM J. LENNARZ received his B.S. in Chemistry from Pennsylvania State University and a Ph.D. in Organic Chemistry from the University of Illinois. Subsequently he carried out postdoctoral work at Harvard with Konrad Bloch on fatty acid biosynthesis. In 1962 he was appointed Assistant Professor at Johns Hopkins in the Department of Physiological Chemistry. After promotion to Associate Professor in 1967, and full Professor in 1971, he remained at Hopkins until 1983. At that time, he was appointed Robert A. Welch Professor and Chair of the Department of Biochemistry and Molecular Biology at the University of Texas Cancer Center, M.D. Anderson Hospital. In 1989 he became a Leading Professor and Chair of the Department of Biochemistry and Cell Biology at SUNY at Stony Brook. In 1990 he founded and became Director of the Institute for Cell and Developmental Biology at Stony Brook.

Dr. Lennarz has served on many national and international committees. He has served as President of the Biochemistry Chairman's Organization, President of the American Society for Biochemistry and Molecular Biology and President of the Society for Glycobiology. He was a member of the Executive Committee of the International Union of Biochemistry and Molecular Biology for almost a decade.

He has presented special lectures at the University of Notre Dame, the NIH, the University of West Virginia, Johns Hopkins University, Florida State University, the University of California at San Diego, the University of Arkansas, Indiana University and the Medical College of Virginia.

He is a member of the National Academy of Sciences. The focus of his early work was on lipids and bacterial cell surfaces. More recent efforts have been in the structure, biosynthesis and function of cell surface glycoproteins. The biosynthesis studies initially were carried out in liver and oviduct, but these efforts now are focused in yeast. The functional studies have concentrated on the role of cell surface glycoproteins in fertilization and early development in the sea urchin and, more recently, the frog. For over 30 years Dr. Lennarz' research has been supported by federal sources, primarily the National Institutes of Health. Recently he was appointed Distinguished Professor and Chair of his department.


## M. Daniel Lane

The Johns Hopkins University, School of Medicine, Baltimore, Maryland, USA

***Section: Metabolism, Vitamins and Hormones***


M. DANIEL LANE received B.S. and M.S. degrees in 1951 and 1953 from Iowa State University and a Ph.D. in 1956 from the University of Illinois. He was a Senior Postdoctoral Fellow with Professor Feodor Lynen at the Max-Planck Institute Fur Zellchemie in Munich. Following faculty positions at Virginia Polytechnic Institute and New York University School of Medicine, he joined the faculty at the Johns Hopkins University School of Medicine in 1969 and served as DeLamar Professor and Director of the Department of Biological Chemistry from 1978 to 1997. He is presently Distinguished Service Professor at Johns Hopkins. In 2002 he received an honorary degree, Doctor of Humane Letters, from Iowa State University.

Dr. Lane was elected to membership in the National Academy of Sciences (in 1987) and was elected as a Fellow of the American Academy of Arts and Sciences (in 1982) and of the American Society of Nutritional Sciences (in 1996). He received the Mead Johnson Award from the American Society for Nutritional Sciences in 1966 for his research on biotin-dependent enzymes and in 1981, the William C. Rose Award from the American Society for Biochemistry and Molecular Biology for his work on the insulin receptor. In 1990–1991 Lane served as President of the American Society of Biochemistry and Molecular Biology. He has presented many named lectureships (including the

Feodor Lynen Lecture in Germany in 1999) and served on numerous editorial boards including the Journal of Biological Chemistry and the Annual Reviews of Biochemistry. Currently he is Associate Editor for Biochemical and Biophysical Research Communications.

Dr. Lane has published 280 research papers in major scientific journals. His early work focused on various enzymatic $CO_2$ fixation reactions, notably the mechanisms by which the B-vitamin, biotin, functions in enzymes to catalyze carboxylation. Dr. Lane's work on the regulation of acetyl-CoA carboxylase, the key regulatory enzyme of fatty acid synthesis, led him to his present interests which are to understand the basic mechanisms of lipogenesis, adipogenesis and the consequence of aberrations in these processes, most notably obesity. Research currently underway in his laboratory focuses on: (1) the genes that signal stem cell "commitment" to the adipocyte lineage and subsequent differentiation into adipocytes, and (2) the mechanisms by which the region of the brain, known as the hypothalamus, monitors and controls the drive to eat.

# Associate Editors

## Ernesto Carafoli

Università degli Studi di Padova, Padova, Italy
***Section: Bioenergetics***

ERNESTO CARAFOLI earned his M.D. degree at the University of Modena in Italy in 1957. After postdoctoral studies in the Laboratory of Albert L. Lehninger at Johns Hopkins University in the mid 1960s he returned to his home institution in Italy where he worked until 1973, when he was appointed Professor of Biochemistry at the Swiss Federal Institute of Technology (ETH) in Zurich. He returned to Italy in 1998 as a Professor of Biochemistry at the University of Padova, where he now also directs the newly founded Venetian Institute of Molecular Medicine (VIMM).

Dr. Carafoli became interested in calcium as a signaling agent during his post-doctoral days at Johns Hopkins. When he arrived there his main interests were in mitochondrial bioenergetics and it was thus natural for him to expand them to the newly discovered area of mitochondrial calcium transport. He was involved in most of the early discoveries in the field, and he continued to work on mitochondria and calcium after his return to Italy and until he moved to the ETH. There his interests still remained focused on calcium, but the emphasis shifted to the proteins that transport it across membranes and to those that process its signal. His favorite object of study became the calcium pumps, especially that of the plasma membrane, an enzyme which is essential to the regulation of calcium homeostasis and thus to the well being of cells. His contributions on the enzyme, especially after he purified it in 1979, have helped establishing most of its properties and have clarified important problems of mechanism, regulation and structure.

Dr. Carafoli has authored or co-authored about 450 peer-reviewed articles and reviews, and has edited or co-edited about 20 books. He has served on the Editorial or Advisory Boards of several periodicals and has organized about 30 International Workshops and Symposia. He has been featured as a plenary or honorary lecturer at numerous events ranging from specialized Workshops to International Symposia and Congresses. Dr. Carafoli's honors and awards include several international prizes and medals, memberships in several Academies, and three honorary degrees.

## Don W. Cleveland

University of California, San Diego, La Jolla, CA, USA
***Section: Cell Architecture and Function***

DON W. CLEVELAND has been a longstanding contributor to the elucidation of regulation of assembly of mitotic spindles and chromosome movement and how errors in these contribute to the chromosome loss characteristic of human tumors. He discovered the tubulin gene families encoding the major subunits of microtubules and the first mammalian example of control of gene expression through regulated RNA instability. He identified components required for microtubule nucleation and anchoring during spindle assembly. He identified the first human centromeric protein (CENP-B). He then discovered CENP-E, the centromere-associated, microtubule-motor that he showed to be essential for chromosome attachment and for activation and silencing of the mitotic checkpoint, the cell cycle control mechanism that prevents errors of chromosome segregation in mitosis.

Dr. Cleveland has also been a leading force in dissecting the disease mechanism for major human neurodegenerative disorders. He initially purified and characterized tau, the microtubule-associated protein that assembles aberrantly in human dementias including Alzheimer's disease and Pick's disease. He established that the extreme asymmetry of neurons acquired during development is achieved with a deformable array of interlinked neurofilaments, microtubules and actin. He showed that disorganization of neurofilament arrays caused selective death of motor neurons in mice and humans. He also demonstrated that neuronal death could also arise by a toxicity of mutant superoxide dismutase unrelated to its normal activity, thereby uncovering the mechanism underlying the major genetic form of amyotrophic lateral sclerosis. He showed that this toxicity could be

sharply ameliorated by lowering the content of neurofilaments.

Dr. Cleveland is currently Head, Laboratory for Cell Biology in the Ludwig Institute for Cancer Research and Professor of Medicine, Neurosciences and Cellular and Molecular Medicine at the University of California at San Diego. He is also the Editor of the Journal of Cell Biology and Current Opinion in Cell Biology.

## Jack E. Dixon

University of California, San Diego School of Medicine,
La Jolla, CA, USA
***Section: Protein/Enzyme Structure, Function, and Degradation***

JACK E. DIXON earned his Ph.D. in Chemistry at the University of California, Santa Barbara in 1971 and did his postdoctoral training in Biochemistry at the University of California, San Diego.

Dr. Dixon is a pioneer and leader in the structure and function of the protein tyrosine phosphatases (PTPases). He demonstrated that the unique catalytic mechanism of the PTPases proceeds via a novel cysteine-phosphate intermediate. He discovered the first dual-specificity phosphatase, which led to the identification of the cell cycle protein, $p80^{cdc25}$, as a phosphatase. He also showed that the bacteria responsible for the plague or "black death" harbor the most active PTPase ever described. He and his colleagues went on to demonstrate that this PTPase gene product is essential for the pathogenesis of the bacteria. Dr. Dixon and his colleagues determined X-ray structures for both tyrosine and dual specificity phosphatases. Dr. Dixon also found that sequences outside of the PTPase catalytic domain could function to direct the subcellular localization of the PTPases and to restrict their substrate specificity. This is now a widely acknowledged regulatory paradigm for the PTPases. Recently, his laboratory demonstrated that the tumor suppressor gene, PTEN, which shares sequence identity with the PTPases, catalyzes the dephosphorylation of a lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3). This represents the first example of a PTPase dephosphorylating a lipid second messenger. PIP3 activates the protein kinase, AKT, which plays a critical role in controlling the balance between apoptosis and cell survival. The loss of the PTEN gene elevates PIP3 levels leading to constitutive activation by AKT and oncogenesis. Recently, Dr. Dixon in collaboration with Nikola Pavletich determined the X-ray structure of PTEN. Their structure–function studies explain the PIP3 substrate specificity of PTEN and also provide a rationale for many of the mutations seen in human cancers. Earlier in his career, Dr. Dixon adopted the tools of molecular biology as they became available in the 1970s, and his laboratory was among the first to use synthetic oligonucleotides to isolate and extensively characterize cDNAs encoding peptide hormones.

Dr. Dixon is Professor of Pharmacology, Cellular and Molecular Medicine and Chemistry and Biochemistry and Dean of Scientific Affairs at the University of California, San Diego. He is a member of the National Academy of Sciences, the Institute of Medicine and the American Academy of Arts and Sciences. Dr. Dixon was the recipient of the 2003 William C. Rose Award from the American Society for Biochemistry and Molecular Biology.

## John H. Exton

Howard Hughes Medical Institute, Vanderbilt University School of Medicine, Nashvillie, TN, USA
***Section: Signaling***

JOHN H. EXTON was born and educated in New Zealand where he received his medical training and a Ph.D. in Biochemistry from the University of Otago in 1963. He did postdoctoral work at Vanderbilt University under Charles R. Park and Earl W. Sutherland, and became an Investigator of the Howard Hughes Medical Institute in 1968 and Professor of Physiology in 1970. He is presently Professor of Molecular Physiology and Biophysics, Professor of Pharmacology and a Hughes Investigator at Vanderbilt.

Dr. Exton's research initially focused on the changes in carbohydrate metabolism in liver during diabetes and treatment with various hormones using the perfused rat liver as the experimental system. His work concentrated on gluconeogenesis and identified the enzymatic reactions that were under control by insulin, epinephrine (adrenaline), glucagon and glucocorticoids, and demonstrated the importance of cyclic AMP in the regulation of these reactions. The role played by the supply of substrates, especially of alanine, was also shown.

Dr. Exton then turned his attention to the action of epinephrine (adrenaline) and demonstrated that many of its actions were not mediated by cyclic AMP but by calcium ions. This led to study of the breakdown of inositol phospholipids by phospholipase C that underlay the increase in calcium. Later this resulted in the discovery of $G_q$, a novel G protein that activated phospholipase C. Further studies demonstrated that agonists caused the breakdown of another phospholipid (phosphatidylcholine) by another phospholipase (phospholipase D). Current work is focused on the physiological role of phospholipase D.

Dr. Exton has authored over 350 scientific articles and is presently an Associate Editor of the Journal of Biological Chemistry. He has served on many scientific review groups and as a reviewer for many journals. He has won numerous awards, most notably the Lilly

Award of the American Diabetes Association, Fellow of the American Association for the Advancement of Science and election to membership in the National Academy of Sciences.

## Paul Modrich

Duke University Medical Center, Durham, NC, USA
***Section: Molecular Biology***

PAUL MODRICH is an Investigator of the Howard Hughes Medical Institute and James B. Duke Professor of Biochemistry at Duke University Medical Center. He received his undergraduate degree from M.I.T. and his Ph.D. in Biochemistry from Stanford University. His current research addresses the mechanisms of DNA repair. He has served on the editorial boards of the Journal of Biological Chemistry, Biochemistry, Proceedings of the National Academy of Sciences, and DNA Repair. His honors include election to National Academy of Sciences and the Institute of Medicine, the Pfizer Award in Enzyme Chemistry, the General Motors Mott Prize in Cancer Research, and the Pasarow Foundation Award in Cancer Research.

# Preface

Biological Chemistry is defined as the chemistry of the compounds and processes that constitute living organisms. The ultimate goal, of course, is to understand and define biology at a mechanistic level. This was aptly stated in an historical treatise on the founding of the *Journal of Biological Chemistry*, where John Edsall quoted a statement in a letter from J. L. Loeb (in Berkeley), "The future of biology lies with those who attack its problems from a chemical point of view." What was an emerging field in 1900 with its origins in physiology, nutrition and chemistry has broadened and expanded to include numerous other fields including mechanistic enzymology, molecular biology, structural biology, cell biology, genomics, proteomics, bioinformatics, metabolomics and others, that were not defined as discrete fields at that time.

Modern biochemistry (biological chemistry) began with the accidental discovery by Eduard Buchner in 1897 that a cell-free yeast extract could carry out fermentation of glucose to alcohol and $CO_2$ *in the absence of intact cells*. He named the dissolved substance responsible for this process zymase, the substance(s) we now refer to as enzymes. Importantly, Buchner recognized the significance of his discovery. This ended the dogma of the time, perpetuated by Pasteur, the concept of *vitalism*; i.e., that fermentation (and presumably other complex biological phenomena) required the action of intact cells. Thus, serendipity and a prepared mind ushered in a new era of discovery. Now it became possible to dissect complex physiological processes and to study them with preparations free of the constraints of intact cells. Once a metabolic pathway/process was established, it became possible to purify the enzymes, cofactors and substrates involved, to reconstitute the process with purified components and to characterize the components chemically. What followed was an information explosion in the field of biochemistry and progression through a series of trends, each "in vogue" in its time. The identification of the dietary essentials, the hunt for the vitamins/cofactors, the hormones, identification of metabolic pathways and the enzymes involved, oxidative phosphorylation, protein synthesis, molecular biology—each developed as a primary focus.

The need to associate chemistry with function came early and was evident in the naming of departments and journals. Over time names changed from Agricultural Chemistry to Physiological Chemistry to Biochemistry to Biological Chemistry. An example is the Department of Biochemistry at the University of Wisconsin, which began in 1883 as the Department of Agricultural Chemistry.

Where are we headed? We have reached the point where the borders of these areas have become blurred. What constitutes cell biology, molecular biology/genetics, developmental biology, physiology, immunology—ultimately reduces to chemistry. To understand these processes we must know what the molecules are and understand how they interact, i.e. the basic chemistry. That is what this encyclopedia is about.

The breadth of content of this encyclopedia aims to cover major topics of modern biochemistry, each authored by an expert in the area. We feel that the coverage is broad and we have been inclusive in choice of topics. The encyclopedia is a reference work encompassing four volumes containing over 500 articles with more than 750 authors or coauthors. Each article/topic covers an important area of the field which reflects the point of view of the authors. Together the articles cover virtually every aspect of biology for which we have "mechanistic" information. For those who wish to probe more deeply into a topic, references to further readings are included at the end of each article. The editorial board that made decisions on coverage consists of seven members, each an expert representing a major area in the field of biochemistry. A dedicated effort was made to provide coverage that is as complete as possible. The content is presented at a level that we hope will be interpretable to interested individuals with some background in chemistry and biology. It is intended for such individuals rather than specialists with extensive scientific backgrounds in specific areas. It is aimed at the generalist as opposed to the specialist.

Finally, we would like to single out Gail Rice and Dr. Noelle Gracy for their enormous contribution in putting this encyclopedia together. They, in fact, were a driving force that brought this major work to completion.



# Notes on the Subject Index

Abbreviations used in subentries without explanation:

| | |
|---|---|
| CoA | coenzyme A |
| DAG | diacylglycerol |
| ELISA | enzyme-linked immunosorbent assay |
| ERK | extracellular-signal regulated kinase |
| GlcNAC | N-Acetylglucosamine |
| HPLC | high-pressure liquid chromatography |
| $IP_3$ | inositol 1,4,5-triphosphate |
| MAP | mitogen-activated protein |
| MMP | matrix metalloproteinase |
| mtDNA | mitochondrial DNA |
| NADPH | nicotinamide-adenine dinucleotide phosphate |
| PFK-2/ FBPase-2 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase |
| PI3K | phosphatidylinositol 3-kinase |
| $PIP_2$ | phosphatidylinositol 4,5-bisphosphate |
| $PIP_3$ | phosphatidylinositol-3,4,5-triphosphate |
| PPAR | peroxisome proliferator-activated receptor |
| RPLC | reversed-phase high-performance liquid chromatography |

**Volume 1**

ABC Transporters, Pages 1-5, Andre Goffeau, Benoit De Hertogh and Philippe V. Baret

Abscisic Acid (ABA), Pages 6-11, Ramanjulu Sunkar and Jian-Kang Zhu

Actin Assembly/Disassembly, Pages 12-18, Henry N. Higgs

Actin-Capping and -Severing Proteins, Pages 19-26, Sankar Maiti and James R. Bamburg

Actin-Related Proteins, Pages 27-33, R. Dyche Mullins

Adenosine Receptors, Pages 34-39, Lauren J. Murphree and Joel Linden

Adenylyl Cyclases, Pages 40-45, Ronald Taussig

Adrenergic Receptors, Pages 46-50, David B. Bylund

Affinity Chromatography, Pages 51-56, Pedro Cuatrecasas and Meir Wilchek

Affinity Tags for Protein Purification, Pages 57-63, Joseph J. Falke and John A. Corbin

A-Kinase Anchoring Proteins, Pages 64-67, Lorene K. Langeberg and John D. Scott

Allosteric Regulation, Pages 68-73, Barry S. Cooperman

Alternative Splicing: Regulation of Fibroblast Growth Factor Receptor (FGFR), Pages 74-77, Mariano A. Garcia-Blanco

Alternative Splicing: Regulation of Sex Determination in Drosophila melanogaster, Pages 78-84, Jill K. M. Penn, Patricia Graham and Paul Schedl

Amine Oxidases, Pages 85-89, Giovanni Floris and Alessandro Finazzi Agro

Amino Acid Metabolism, Pages 90-95, Luc Cynober

Aminopeptidases, Pages 96-98, Ralph A. Bradshaw

Amyloid, Pages 99-104, Ronald Wetzel

Anaplerosis, Pages 105-110, Raymond R. Russell, III and Heinrich Taegtmeyer

Angiotensin Receptors, Pages 111-115, Tadashi Inagami

ara Operon, Pages 116-119, Robert F. Schleif

ARF Family, Pages 120-122, Gustavo Pacheco-Rodriguez, Joel Moss and Martha Vaughan

Aspartic Proteases, Pages 123-127, Ben M. Dunn

ATP Synthesis in Plant Mitochondria: Substrates, Inhibitors, Uncouplers, Pages 128-132, Kathleen L. Soole and R. Ian Menz

ATP Synthesis: Mitochondrial Cyanide-Resistant Terminal Oxidases, Pages 133-137, James N. Siedow

Autophagy in Fungi and Mammals, Pages 138-143, Daniel J. Klionsky and Ju Guan

B12-Containing Enzymes, Pages 145-151, Vahe Bandarian and Rowena G. Matthews

Bax and Bcl2 Cell Death Enhancers and Inhibitors, Pages 152-154, David L. Vaux

B-Cell Antigen Receptor, Pages 155-158, Thomas M. Yankee and Edward A. Clark

Bile Salts and their Metabolism, Pages 159-163, Ulrich Beuers and Thomas Pusl

Biliary Cirrhosis, Primary, Pages 164-169, Marshall M. Kaplan

Bioenergetics: General Definition of Principles, Pages 170-173, David G. Nicholls

Biotin, Pages 174-178, Steven W. Polyak and Anne Chapman-Smith

Biotinylation of Proteins, Pages 179-181, Ronald A. Kohanski

Bradykinin Receptors, Pages 182-185, Ronald M. Burch

Branched-Chain -Ketoacids, Pages 186-191, David T. Chuang

Brassinosteroids, Pages 192-197, Steven D. Clouse

Cadherin Signaling, Pages 199-204, David B. Sacks and Jonathan M. G. Higgins

Cadherin-Mediated Cell-Cell Adhesion, Pages 205-211, Frauke Drees and W. James Nelson

Calcitonin Gene-Related Peptide and Adrenomedullin Receptors, Pages 212-216, Debbie L. Hay, Alex C. Conner and David R. Poyner

Calcitonin Receptor, Pages 217-220, Samia I. Girgis, Niloufar Moradi-Bidhendi, Lucia Mancini and Iain MacIntyre

Calcium Buffering Proteins: Calbindin, Pages 221-225, Willi Hunziker and Igor Bendik

Calcium Buffering Proteins: ER Luminal Proteins, Pages 226-230, Jody Groenendyk and Marek Michalak

Calcium Oscillations, Pages 231-234, Marisa Brini

Calcium Sensing Receptor, Pages 235-240, Jacob Tfelt-Hansen and Edward M. Brown

Calcium Signaling: Calmodulin-Dependent Phosphatase, Pages 241-245, Claude Klee, Hao Ren and Shipeng Li

Calcium Signaling: Cell Cycle, Pages 246-249, Luigia Santella

Calcium Signaling: Motility (Actomyosin-Troponin System), Pages 250-255, Takeyuki Wakabayashi and Setsuro Ebashi

Calcium Signaling: NO Synthase, Pages 256-260, Zhi-Qiang Wang and Dennis J. Stuehr

Calcium Transport in Mitochondria, Pages 261-266, Rosario Rizzuto and Marisa Brini

Calcium Waves, Pages 267-269, Lionel F. Jaffe

Calcium, Biological Fitness of, Pages 270-273, Robert J. P. Williams

Calcium/Calmodulin-Dependent Protein Kinase II, Pages 274-280, Andy Hudmon and Howard Schulman

Calcium/Calmodulin-Dependent Protein Kinases, Pages 281-286, J. Robison and Roger J. Colbran

Calcium-Binding Proteins: Cytosolic (Annexins, Gelsolins, C2-Domain Proteins) , Pages 287-293, Joachim Krebs

Calcium-Modulated Proteins (EF-Hand), Pages 294-299, Robert H. Kretsinger

Calpain, Pages 300-306, Hiroyuki Sorimachi and Yasuko Ono

Carbohydrate Chains: Enzymatic and Chemical Synthesis, Pages 307-313, Thomas J. Tolbert and Chi-Huey Wong

Carnitine and -Oxidation, Pages 314-318, Janos Kerner and Charles L. Hoppel

Caspases and Cell Death, Pages 319-327, Don W. Nicholson, Pierluigi Nicotera and Gerry Melino

Cell Cycle Controls in G1 and G0, Pages 328-331, WengeShi and Steven F. Dowdy

Cell Cycle: Control of Entry and Progression Through S Phase, Pages 332-337, Susan L. Forsburg

Cell Cycle: DNA Damage Checkpoints, Pages 338-344, Jean Y. J. Wang

Cell Cycle: Mitotic Checkpoint, Pages 345-351, Tim J. Yen

Cell Death by Apoptosis and Necrosis, Pages 352-355, Pierluigi Nicotera

Cell Migration, Pages 356-361, J. Victor Small and Emmanuel Vignal

Cell-Matrix Interactions, Pages 362-366, Janet A. Askari and Martin J. Humphries

Centromeres, Pages 367-371, Beth A. Sullivan

Centrosomes and Microtubule Nucleation, Pages 372-376, Reiko Nakajima, Ming-Ying Tsai and Yixian Zheng

c-fes Proto-Oncogene, Pages 377-382, Thomas E. Smithgall and Robert I. Glazer

Chaperones for Metalloproteins, Pages 383-386, Valeria C. Culotta and Edward Luk

Chaperones, Molecular, Pages 387-392, Sue Wickner and Joel R. Hoskins

Chaperonins, Pages 393-398, Arthur L. Horwich, Wayne A. Fenton and George W. Farr

Chemiluminescence and Bioluminescence, Pages 399-404, Thomas O. Baldwin

Chemiosmotic Theory, Pages 405-412, Keith D. Garlid

Chemokine Receptors, Pages 413-418, Ann Richmond and Guo-Huang Fan

Chemolithotrophy, Pages 419-424, Alan B. Hooper

Chemotactic Peptide/Complement Receptors, Pages 425-429, Eric R. Prossnitz and Larry A. Sklar

Chlorophylls and Carotenoids, Pages 430-437, Hugo Scheer

Chloroplast Redox Poise and Signaling, Pages 438-445, John F. Allen

Chloroplasts, Pages 446-450, Nicoletta Rascio

Cholesterol Synthesis, Pages 451-455, Peter A. Edwards

Chromatin Remodeling, Pages 456-463, Eric Kallin and Yi Zhang

Chromatin: Physical Organization, Pages 464-468, Christopher L. Woodcock

Chromosome Organization and Structure, Overview, Pages 469-474, Elena Gracheva and Sarah C. R. Elgin

Coenzyme A, Pages 475-477, M. Daniel Lane

Collagenases, Pages 478-481, Kenn Holmbeck and Henning Birkedal-Hansen

Collagens, Pages 482-487, Darwin J. Prockop

Cyclic AMP Receptors of Dictyostelium, Pages 488-493, Dale Hereld and Peter N. Devreotes

Cyclic GMP Phosphodiesterases, Pages 494-500, Sharron H. Francis and Jackie D. Corbin

Cyclic Nucleotide Phosphodiesterases, Pages 501-505, Vincent C. Manganiello and Eva Degerman

Cyclic Nucleotide-Dependent Protein Kinases, Pages 506-511, Sharron H. Francis and Jackie D. Corbin

Cyclic Nucleotide-Regulated Cation Channels, Pages 512-515, Martin Biel and Franz Hofmann

Cysteine Proteases, Pages 516-520, David J. Buttle and John S. Mort

Cytochrome b6f Complex, Pages 521-527, Gunter A. Hauska and Thomas Schodl

Cytochrome bc1 Complex (Respiratory Chain Complex III), Pages 528-534, Bernard L. Trumpower

Cytochrome c, Pages 535-538, Hans Tuppy and Gunther Kreil

Cytochrome Oxidases, Bacterial, Pages 539-543, Peter Brzezinski and Pia Adelroth

Cytochrome P-450, Pages 544-549, Rita Bernhardt

Cytokines, Pages 550-555, Andrea L. Wurster and Michael J. Grusby

Cytokinesis, Pages 556-561, Masanori Mishima and Michael Glotzer

Cytokinin, Pages 562-567, Thomas Schmulling

Desmosomes and Hemidesmosomes, Pages 569-576, Rachel L. Dusek, Jonathan C. R. Jones and Kathleen J. Green

Detergent Properties, Pages 577-581, Darrell R. McCaslin

Diabetes, Pages 582-592, David W. Cooke

Diacylglycerol Kinases and Phosphatidic Acid Phosphatases, Pages 593-597, Stephen M. Prescott and Matthew K. Topham

Disulfide Bond Formation, Pages 598-602, Hiram F. Gilbert

DNA Base Excision Repair, Pages 603-608, Hilde Nilsen and Tomas Lindahl

DNA Damage: Alkylation, Pages 609-613, Anton B. Guliaev and B. Singer

DNA Glycosylases: Mechanisms, Pages 614-617, Daniel J. Krosky and James T. Stivers

DNA Helicases: Dimeric Enzyme Action, Pages 618-623, Timothy M. Lohman

DNA Helicases: HexamericEnzyme Action, Pages 624-631, Smita S. Patel

DNA Ligases: Mechanism and Functions, Pages 632-636, Alan E. Tomkinson and John B. Leppard

DNA Ligases: Structures, Pages 637-643, C. Kiong Ho, Mark Odell and Dimitar B. Nikolov

DNA Methyltransferases, Bacterial, Pages 644-651, Albert Jeltsch and Richard I. Gumport

DNA Methyltransferases, Structural Themes, Pages 652-659, Sanjay Kumar

DNA Methyltransferases: Eubacterial GATC, Pages 660-664, Martin G. Marinus

DNA Mismatch Repair and Homologous Recombination, Pages 665-670, Ivan Matic and Miroslav Radman

DNA Mismatch Repair and the DNA Damage Response, Pages 671-674, Guo-Min Li and Steven R. Presnell

DNA Mismatch Repair Defects and Cancer, Pages 675-681, Richard D. Kolodner

DNA Mismatch Repair in Bacteria, Pages 682-686, A-Lien Lu

DNA Mismatch Repair in Mammals, Pages 687-690, James T. Drummond

DNA Mismatch Repair: E. coli Vsr and Eukaryotic G-T Systems, Pages 691-693, Margaret Lieb

DNA Oxidation, Pages 694-697, Arthur P. Grollman and Dmitry O. Zharkov

DNA Photolyase, Pages 698-702, Carrie L. Partch and Aziz Sancar

DNA Polymerase, Eukaryotic $\alpha$, Pages 703-707, Teresa S. -F. Wang

DNA Polymerase, Eukaryotic $\beta$, Pages 708-712, William A. Beard and Samuel H. Wilson

DNA Polymerase, Eukaryotic $\delta$, Pages 713-715, Antero G. So and Kathleen M. Downey

DNA Polymerase, Eukaryotic $\varepsilon$, Pages 716-719, Yasuo Kawasaki and Akio Sugino

DNA Polymerase I, Bacterial, Pages 720-725, Catherine M. Joyce

DNA Polymerase II, Bacterial, Pages 726-728, Judith L. Campbell

DNA Polymerase III, Bacterial, Pages 729-733, Hisaji Maki

DNA Polymerases: Kinetics and Mechanism, Pages 734-739, Kenneth A. Johnson

DNA Replication Fork, Bacterial, Pages 740-744, Nancy G. Nossal

DNA Replication Fork, Eukaryotic, Pages 745-748, Lori M. Kelman, Jerard Hurwitz and Zvi Kelman

DNA Replication, Mitochondrial, Pages 749-752, David A. Clayton

DNA Replication: Eukaryotic Origins and the Origin Recognition Complex, Pages 753-760, Melvin L. DePamphilis and Cong-jun Li

DNA Replication: Initiation in Bacteria, Pages 761-766, Jon M. Kaguni

DNA Restriction and Modification: Type I Enzymes, Pages 767-771, David T. F. Dryden

DNA Restriction and Modification: Type II Enzymes, Pages 772-777, Darren M. Gowers and Stephen E. Halford

DNA Restriction and Modification: Type III Enzymes, Pages 778-781, Desirazu N. Rao and S. Srivani

DNA Secondary Structure, Pages 782-787, Albino Bacolla and Robert D. Wells

DNA Sequence Recognition by Proteins, Pages 788-793, Arabela A. Grigorescu and John M. Rosenberg

DNA Supercoiling, Pages 794-797, Tao-shih Hsieh

DNA Topoisomerases: Type I, Pages 798-805, James J. Champoux

DNA Topoisomerases: Type II, Pages 806-811, Renier Velez-Cruz and Neil Osheroff

DNA Topoisomerases: Type III-RecQ Helicase Systems, Pages 812-816, Rodney Rothstein and Erika Shor

Dopamine Receptors, Pages 817-822, Kim A. Neve

Dynactin, Pages 823-826, Trina A. Schroer

Dynein, Pages 827-831, K. Kevin Pfister

## Volume 2

EF-G and EF-Tu Structures and Translation Elongation in Bacteria, Pages 1-5, Poul Nissen and Jens Nyborg

Eicosanoid Receptors, Pages 6-9, Richard M. Breyer and Matthew D. Breyer

Elastin, Pages 10-12, Judith Ann Foster

Endocannabinoids, Pages 13-15, Daniele Piomelli

Endocytosis, Pages 16-19, Julie G. Donaldson

Endoplasmic Reticulum-Associated Protein Degradation, Pages 20-23, Maurizio Molinari

Energy Transduction in Anaerobic Prokaryotes, Pages 24-30, Gottfried Unden

Enzyme Inhibitors, Pages 31-37, Vern L. Schramm

Enzyme Kinetics, Pages 38-44, Irwin H. Segel

Enzyme Reaction Mechanisms: Stereochemistry, Pages 45-50, Ming-Daw Tsai, Li Zhao and Brandon J. Lamarche

Epidermal Growth Factor Receptor Family, Pages 51-55, Denis Tvorogov and Graham Carpenter

ER/SR Calcium Pump: Function, Pages 56-60, Giuseppe Inesi

ER/SR Calcium Pump: Structure, Pages 61-65, Chikashi Toyoshima and Yuji Sugita

Exonucleases, Bacterial, Pages 66-72, Susan T. Lovett

F1-F0 ATP Synthase, Pages 73-79, Donata Branca

FAK Family, Pages 80-84, Steven K. Hanks

Fat Mobilization: Perilipin and Hormone-Sensitive Lipase, Pages 85-89, Constantine Londos and Alan R. Kimmel

Fatty Acid Oxidation, Pages 90-94, Horst Schulz

Fatty Acid Receptors, Pages 95-98, Christer Owman and Bjorn Olde

Fatty Acid Synthesis and its Regulation, Pages 99-103, Steven D. Clarke and Manabu T. Nakamura

Ferredoxin, Pages 104-106, Giuliana Zanetti and Vittorio Pandini

Ferredoxin-NADP+ Reductase, Pages 107-111, Giuliana Zanetti and Alessandro Aliverti

Fibroblast Growth Factor Receptors and Cancer-Associated Perturbations, Pages 112-117, Marko Kornmann and Murray Korc

Flavins, Pages 118-122, Barrie Entsch and David P. Ballou

Flippases, Pages 123-127, Charles J. Waechter

Focal Adhesions, Pages 128-133, Eli Zamir and Benjamin Geiger

Free Radicals, Sources and Targets of: Mitochondria, Pages 134-142, Alberto Boveris and Enrique Cadenas

Friedreich's Ataxia, Pages 143-145, Paul E. Hart and Anthony H. V. Schapira

G Protein Signaling Regulators, Pages 147-151, John H. Exton

G Protein-Coupled Receptor Kinases and Arrestins, Pages 152-157, Jeffrey L. Benovic

G12/G13 Family, Pages 158-161, Stefan Offermanns

GABAA Receptor, Pages 162-166, Richard W. Olsen and Gregory W. Sawyer

GABAB Receptor, Pages 167-170, S. J. Enna

Galectins, Pages 171-174, R. Colin Hughes

Genome-Wide Analysis of Gene Expression, Pages 175-180, Karine G. Le Roch and Elizabeth A. Winzeler

Gi Family of Heterotrimeric G Proteins, Pages 181-185, Maurine E. Linder

Giant Mitochondria (Megamitochondria), Pages 186-188, Bernard Tandler and Charles L. Hoppel

GlcNAc Biosynthesis and Function, O-Linked, Pages 189-192, Kaoru Sakabe and Gerald W. Hart

Glucagon Family of Peptides and their Receptors, Pages 193-196, Laurie L. Baggio and Daniel J. Drucker

Gluconeogenesis, Pages 197-203, Richard W. Hanson and Oliver E. Owen

Glucose/Sugar Transport in Bacteria, Pages 204-207, Lan Guan and H. Ronald Kaback

Glucose/Sugar Transport in Mammals, Pages 208-212, Silvia Mora and Jeffrey Pessin

Glutamate Receptors, Ionotropic, Pages 213-219, Derek B. Scott and Michael D. Ehlers

Glutamate Receptors, Metabotropic, Pages 220-223, P. Jeffrey Conn

Glutathione Peroxidases, Pages 224-228, Fulvio Ursini and Matilde Maiorino

Glycation, Pages 229-236, Suzanne R. Thorpe and John W. Baynes

Glycine Receptors, Pages 237-243, Bodo Laube and Heinrich Betz

Glycogen Metabolism, Pages 244-248, Peter J. Roach

Glycogen Storage Diseases, Pages 249-254, George H. Sack, Jr.

Glycogen Synthase Kinase-3, Pages 255-260, James R. Woodgett

Glycolipid-Dependent Adhesion Processes, Pages 261-265, Senitiroh Hakomori

Glycolysis, Overview, Pages 266-271, Robert A. Harris

Glycoprotein Folding and Processing Reactions, Pages 272-276, Armando J. Parodi

Glycoprotein-Mediated Cell Interactions, O-Linked, Pages 277-282, Robert S. Haltiwanger

Glycoproteins, N-Linked, Pages 283-292, Mark A. Lehrman

Glycoproteins, Plant, Pages 293-296, Carolyn J. Schultz

Glycosylation in Cystic Fibrosis, Pages 297-301, Andrew D. Rhim, Thomas F. Scanlin and Mary Catherine Glick

Glycosylation, Congenital Disorders of, Pages 302-307, Hudson H. Freeze

Glycosylphosphatidylinositol (GPI) Anchors, Pages 308-311, Anant K. Menon

Golgi Complex, Pages 312-315, Mark Stamnes

Gq Family, Pages 316-320, Wanling Yang and John D. Hildebrandt

Green Bacteria: Secondary Electron Donor (Cytochromes), Pages 321-324, Hirozo Oh-oka and Robert E. Blankenship

Green Bacteria: The Light-Harvesting Chlorosome, Pages 325-330, John M. Olson

Green Sulfur Bacteria: Reaction Center and Electron Transport, Pages 331-336, Gunter A. Hauska and Thomas Schodl

Gs Family of Heterotrimeric G Proteins, Pages 337-341, Susanne M. Mumby

Heat/Stress Responses, Pages 343-347, Davis T. W. Ng

Hematopoietin Receptors, Pages 348-353, Barbara A. Miller and Joseph Y. Cheung

Heme Proteins, Pages 354-361, Johannes Everse

Heme Synthesis, Pages 362-366, Gloria C. Ferreira

Hepatocyte Growth Factor/Scatter Factor Receptor, Pages 367-371, Selma Pennacchietti and Paolo M. Comoglio

Hexokinases/Glucokinases, Pages 372-377, Emile Van Schaftingen

Histamine Receptors, Pages 378-383, Stephen J. Hill and Jillian G. Baker

HIV Protease, Pages 384-387, Ben M. Dunn

HIV-1 Reverse Transcriptase Structure, Pages 388-392, Kalyan Das, Stefan G. Sarafianos, Eddy Arnold and Stephen H. Hughes

Homologous Recombination in Meiosis, Pages 393-397, Nancy M. Hollingsworth

HPLC Separation of Peptides, Pages 398-403, James D. Pearson

Imaging Methods, Pages 405-410, Gyorgy Szabadkai and Rosario Rizzuto

Immunoglobulin (Fc) Receptors, Pages 411-416, Mark Hogarth

Inorganic Biochemistry, Pages 417-420, Robert J. P. Williams

Inositol Lipid 3-Phosphatases, Pages 421-426, Gregory S. Taylor and Jack E. Dixon

Inositol Phosphate Kinases and Phosphatases, Pages 427-429, Stephen B. Shears

Insulin- and Glucagon-Secreting Cells of the Pancreas, Pages 430-435, Franz M. Matschinsky

Insulin Receptor Family, Pages 436-440, Paul F. Pilch and Jongsoon Lee

Integrin Signaling, Pages 441-445, Lawrence E. Goldfinger and Mark H. Ginsberg

Interferon Receptors, Pages 446-451, Christopher P. Elco and Ganes C. Sen

Intermediate Filament Linker Proteins: Plectin and BPAG1, Pages 452-457, Peter Fuchs and Gerhard Wiche

Intermediate Filaments, Pages 458-464, Kelsie M. Bernot and Pierre A. Coulombe

Intracellular Calcium Channels: cADPR-Modulated (Ryanodine Receptors), Pages 465-468, Antony Galione

Intracellular Calcium Channels: NAADP+-Modulated, Pages 469-472, Armando A. Genazzani and Marcella Debidda

Ion Channel Protein Superfamily, Pages 473-477, William A. Catterall

IP3 Receptors, Pages 478-481, Colin W. TaylorEdward Morris and Paula da Fonseca

Iron-Sulfur Proteins, Pages 482-489, Helmut Beinert

JAK-STAT Signaling Paradigm, Pages 491-496, Edward Cha and Christian Schindler

Keratins and the Skin, Pages 497-504, Pierre A. Coulombe and Kelsie M. Bernot

Ketogenesis, Pages 505-507, Janos Kerner and Charles L. Hoppel

Kinesin Superfamily Proteins, Pages 508-516, Nobutaka Hirokawa and Reiko Takemura

Kinesins as Microtubule Disassembly Enzymes, Pages 517-521, Susan L. Kline-Smith and Arshad Desai

Kinetic Isotope Effects, Pages 522-527, Justine P. Roth and Judith P. Klinman

lac Operon, Pages 529-534, Liskin Swint-Kruse and Kathleen S. Matthews

Lectins, Pages 535-540, Nathan Sharon and Halina Lis

Leptin, Pages 541-545, Thomas W. Gettys

LexA Regulatory System, Pages 546-550, Veronica G. Godoy, Penny J. Beuning and Graham C. Walker

Ligand-Operated Membrane Channels: Calcium (Glutamate), Pages 551-561, Elias K. Michaelis

Ligand-Operated Membrane Channels: GABA, Pages 562-566, F. Minier and Erwin Sigel

Light-Harvesting Complex (LHC) I and II: Pigments and Proteins, Pages 567-570, Stefan Jansson

Lipases, Pages 571-575, Howard L. Brockman

Lipid Bilayer Structure, Pages 576-579, Erwin London

Lipid Modification of Proteins: Targeting to Membranes, Pages 580-583, Marilyn D. Resh

Lipid Rafts, Pages 584-587, Deborah A. Brown

Lipoproteins, HDL/LDL, Pages 588-593, Fayanne E. Thorngate and David L. Williams

Low Barrier Hydrogen Bonds, Pages 594-598, Perry A. Frey

Luft's Disease, Pages 599-601, Salvatore DiMauro

Lysophospholipid Receptors, Pages 602-604, Gabor J. Tigyi

MDR Membrane Proteins, Pages 605-609, Nathan C. Rockwell

Meiosis, Pages 610-616, Neil Hunter

Melanocortin System, Pages 617-620, Roger D. Cone

Membrane Fusion, Pages 621-626, Joshua Zimmerberg and Leonid V. Chernomordik

Membrane Transport, General Concepts, Pages 627-630, Stanley G. Schultz

Membrane Transporters:Na+/Ca2+ Exchangers, Pages 631-636, Jonathan Lytton

Membrane-Associated Energy Transduction in Bacteria and Archaea, Pages 637-645, Gunter Schafer

Metabolite Channeling: Creatine Kinase Microcompartments, Pages 646-651, Uwe Schlattner and Theo Wallimann

Metalloproteases, Pages 652-656, David S. Auld

Metalloproteinases, Matrix, Pages 657-665, Hideaki Nagase and Gillian Murphy

Metaphase Chromosome, Pages 666-671, Sharron Vass and Margarete M. S. Heck

Methyl-CpG-Binding Proteins, Pages 672-675, David G. Skalnik

Microtubule-Associated Proteins, Pages 676-682, Nobutaka Hirokawa and Reiko Takemura

Mitochondrial Auto-Antibodies, Pages 683-688, Harold Baum

Mitochondrial Channels, Pages 689-692, M. Catia Sorgato and Alessandro Bertoli

Mitochondrial DNA, Pages 693-696, Gottfried Schatz

Mitochondrial Genes and their Expression: Yeast, Pages 697-702, Piotr P. Slonimski and Giovanna Carignani

Mitochondrial Genome, Evolution, Pages 703-708, B. Franz Lang, Dennis V. Lavrov and Gertraud Burger

Mitochondrial Genome, Overview, Pages 709-715, Douglas C. Wallace

Mitochondrial Inheritance, Pages 716-719, Eric A. Shoubridge

Mitochondrial Membranes, Structural Organization, Pages 720-724, Carmen A. Mannella

Mitochondrial Metabolite Transporter Family, Pages 725-732, Ferdinando Palmieri and Martin Klingenberg

Mitochondrial Outer Membrane and the VDAC Channel, Pages 733-736, Marco Colombini

Mitogen-Activated Protein Kinase Family, Pages 737-742, Hidemi Teramoto and J. Silvio Gutkind

Mitosis, Pages 743-747, Patricia Wadsworth and Nasser M. Rusan

mRNA Polyadenylation in Eukaryotes, Pages 748-752, Mary Edmonds

mRNA Processing and Degradation in Bacteria, Pages 753-757, Deborah A. Steege

Mucin Family of Glycoproteins, Pages 758-764, Juan Perez-Vilar and Robert L. Hill

Mucins in Embryo Implantation, Pages 765-769, Daniel D. Carson

Multiple Sequence Alignment and Phylogenetic Trees, Pages 770-774, Russell F. Doolittle

Muscarinic Acetylcholine Receptors, Pages 775-777, Neil M. Nathanson

Myosin Motors, Pages 778-781, Roy E. Larson

## Volume 3

Natriuretic Peptides and their Receptors, Pages 1-5, Lincoln R. Potter

N-End Rule, Pages 6-10, Alexander Varshavsky

Neoglycoproteins, Pages 11-15, Y. C. Lee and Reiko T. Lee

Neuronal Calcium Signal, Pages 16-20, Hilmar Bading

Neuronal Intermediate Filaments, Pages 21-25, Ronald K. H. Liem

Neuropeptide Y Receptors, Pages 26-31, Eric M. Parker

Neurotensin Receptors, Pages 32-36, William Rostene, Patrick Kitabgi and Didier Pelaprat

Neurotransmitter Transporters, Pages 37-40, Aurelio Galli, Randy D. Blakely and Louis J. DeFelice

Neurotrophin Receptor Signaling, Pages 41-45, Jennifer J. Gentry and Bruce D. Carter

Nicotinamide Nucleotide Transhydrogenase, Pages 50-56, Jan Rydstrom

Nicotinic Acetylcholine Receptors, Pages 57-61, Nivalda O. Rodrigues-Pinguet and Henry A. Lester

Nitric Oxide Signaling, Pages 62-65, Michael A. Marletta

N-Linked Glycan Processing Glucosidases and Mannosidases, Pages 46-49, Linda O. Tremblay and Annette Herscovics

Non-Homologous End Joining, Pages 66-70, Penny A. Jeggo

Nonhomologous Recombination: Bacterial Transposons, Pages 71-79, Bao Ton Hoang and Michael G. Chandler

Nonhomologous Recombination: Retrotransposons, Pages 80-86, Siew Loon Ooi and Jef D. Boeke

Nuclear Compartmentalization, Pages 87-91, Kelly P. Smith and Jeanne B. Lawrence

Nuclear Envelope and Lamins, Pages 92-95, Bryce M. Paschal

Nuclear Factor kappaB, Pages 96-99, Thomas D. Gilmore

Nuclear Genes in Mitochondrial Function and Biogenesis, Pages 100-104, Alexander Tzagoloff and Carol L. Dieckmann

Nuclear Organization, Chromatin Structure, and Gene Silencing , Pages 105-108, Lori L. Wallrath, John R. Danzer, Oya Yazgan and Pamela K. Geyer

Nuclear Pores and Nuclear Import/Export, Pages 109-114, Anita H. Corbett

Nucleoid Organization of Bacterial Chromosomes, Pages 115-118, Charles J. Dorman

Nucleolus, Overview, Pages 119-122, Thoru Pederson

Nucleotide Excision Repair and Human Disease, Pages 123-129, James E. Cleaver

Nucleotide Excision Repair in Eukaryotes, Pages 130-133, Laura A. Lindsey-Boltz and Aziz Sancar

Nucleotide Excision Repair, Bacterial: The UvrABCD System, Pages 134-142, Bennett Van Houten and Lawrence Grossman

Nucleotide Excision Repair: Biology, Pages 143-147, Errol C. Friedberg

Olfactory Receptors, Pages 149-154, Sigrun I. Korsching

Oligosaccharide Analysis by Mass Spectrometry, Pages 155-160, Andrew J. Hanneman and Vernon N. Reinhold

Oligosaccharide Chains: Free, N-Linked, O-Linked, Pages 161-164, Tadashi Suzuki

Oncocytes, Pages 165-166, Bernard Tandler and Charles L. Hoppel

Opioid Receptors, Pages 167-171, P. Y. Law and Horace H. Loh

Ornithine Cycle, Pages 172-177, Malcolm Watford

Oxygenases, Pages 178-182, Osamu Hayaishi

P2X Purinergic Receptors, Pages 183-187, Annmarie Surprenant

P2Y Purinergic Receptors, Pages 188-191, George R. Dubyak

p53 Protein, Pages 192-195, Jamie Hearnes and Jennifer Pietenpol

p70 S6 Kinase/mTOR, Pages 196-200, Christopher G. Proud

Parathyroid Hormone/Parathyroid Hormone-Related Protein Receptor, Pages 201-207, Thomas J. Gardella

PCR (Polymerase Chain Reaction), Pages 208-210, Michael J. Brownstein

Pentose Phosphate (Hexose Mono Phosphate) Pathway, Pages 211-215, Bernard R. Landau

Pentose Phosphate Pathway, History of, Pages 216-225, John F. Williams

Peptide Amidation, Pages 226-230, Mark J. Niciu, Richard E. Mains and Betty A. Eipper

Periplasmic Electron Transport Systems in Bacteria, Pages 231-238, David J. Richardson, Gary Sawers and Rob J. M. Van Spanning

Peroxisome Proliferator-Activated Receptors, Pages 239-245, Mary C. Sugden, Edward A. Sugden and Mark J. Holness

Peroxisomes, Pages 246-250, Suresh Subramani

Phage Display for Protein Binding, Pages 251-255, Henry B. Lowman

Pheromone Receptors (Yeast), Pages 256-261, James B. Konopka and Jeremy W. Thorner

PHO Regulon, Pages 262-265, Bengt L. Persson

Phosphatidylinositol Bisphosphate and Trisphosphate, Pages 266-271, Alex Toker

Phosphatidylinositol-3-Phosphate, Pages 272-276, Joseph V. Virbasius and Michael P. Czech

Phosphofructokinase-2/Fructose Bisphosphatase-2, Pages 277-280, Daniel M. Raben

Phosphoinositide 3-Kinase, Pages 281-286, Khatereh Ahmadi and Michael Waterfield

Phosphoinositide 4- and 5-Kinases and Phosphatases, Pages 287-291, Shawn F. Bairstow, Matthew W. Bunce and Richard A. Anderson

Phosphoinositide-Dependent Protein Kinases, Pages 292-296, Nick R. Leslie and C. Peter Downes

Phospholipase A2, Pages 297-300, Timothy R. Smith and Edward A. Dennis

Phospholipase C, Pages 301-305, Fujio Sekiya, Yeun Ju Kim and Sue Goo Rhee

Phospholipase D, Pages 306-313, Mary M. LaLonde and Michael A. Frohman

Phospholipid Metabolism in Mammals, Pages 314-320, Claudia Kent

Phospholipid Synthesis in Yeast, Pages 321-325, Gil-Soo Han and George M. Carman

Photoreceptors, Pages 326-329, King-Wai Yau

Photosynthesis, Pages 330-335, Richard C. Leegood

Photosynthetic Carbon Dioxide Fixation, Pages 336-341, Matthew J. Paul

Photosystem I, Structure and Function, Pages 342-347, Petra Fromme

Photosystem I: FX, FA, and FB Iron-Sulfur Clusters, Pages 348-356, Mikhail L. Antonkine and John H. Golbeck

Photosystem II Light Harvesting System: Dynamic Behavior, Pages 357-362, Peter Horton and Alexander Ruban

Photosystem II: Assembly and Turnover of the D1 Protein, Pages 363-366, Eira Kanervo and Eva-Mari Aro

Photosystem II: Protein Components, Pages 367-374, James Barber

Photosystem II: Water Oxidation, Overview, Pages 375-380, Fabrice Rappaport and Pierre Joliot

Plant Signaling: Peptides, Pages 381-384, Clarence A. Ryan and Gregory Pearce

Plasma-Membrane Calcium Pump: Structure and Function, Pages 385-389, Emanuel E. Strehler

Plastocyanin, Pages 390-393, Elizabeth L. Gross

Platelet-Activating Factor Receptor, Pages 394-398, Katherine M. Howard and Merle S. Olson

Platelet-Derived Growth Factor Receptor Family, Pages 399-406, Marina Kovalenko and Andrius Kazlauskas

Polysialic Acid inMolecular Medicine, Pages 407-414, Frederic A. Troy, II

Porphyrin Metabolism, Pages 415-419, Mark Shepherd and Harry A. Dailey

Pre-tRNA and Pre-rRNA Processing in Bacteria, Pages 420-424, Sidney R. Kushner

Pre-tRNA and Pre-rRNA Processing in Eukaryotes, Pages 425-431, Hendrik A. Raue

Prions and Epigenetic Inheritance, Pages 432-436, Reed B. Wickner

Prions, Overview, Pages 437-440, Cedric Govaerts and Fred E. Cohen

Processivity Clamps in DNA Replication: Clamp Loading, Pages 441-446, Megan J. Davey and Mike O'Donnell

Propionyl CoA-Succinyl CoA Pathway, Pages 447-451, Wayne A. Fenton

Prostaglandins and Leukotrienes, Pages 452-456, William L. Smith and Robert C. Murphy

Proteases in Blood Clotting, Pages 457-463, John D. Kulman and Earl W. Davie

Proteasomes, Overview, Pages 464-468, Martin Rechsteiner

26S Proteasome, Structure and Function, Pages 469-473, Peter Zwickl and Wolfgang Baumeister

Protein Carboxyl Esterification, Pages 474-477, Shilpa G. Lalchandani and Jeffry B. Stock

Protein Data Resources, Pages 478-483, Philip E. Bourne

Protein Degradation, Pages 484-492, Tomo ari and Alfred L. Goldberg

Protein Folding and Assembly, Pages 493-499, David P. Goldenberg

Protein Glycosylation Inhibitors, Pages 500-503, Alan D. Elbein

Protein Glycosylation, Overview, Pages 504-509, Natasha E. Zachara and Gerald W. Hart

Protein Import into Mitochondria, Pages 510-515, Johannes M. Herrmann and Walter Neupert

Protein Kinase B, Pages 516-522, Bettina A. Dummler and Brian A. Hemmings

Protein Kinase C Family, Pages 523-526, Alexandra C. Newton

Protein N-Myristoylation, Pages 527-531, Erica Dutil Sonnenburg and Jeffrey I. Gordon

Protein Palmitoylation, Pages 532-535, Robert J. Deschenes

Protein Tyrosine Phosphatases, Pages 536-542, David J. Pagliarini, Fred L. Robinson and Jack E. Dixon

Proteinase-Activated Receptors, Pages 543-548, Kristina K. Hansen and Morley D. Hollenberg

Proteoglycans, Pages 549-555, Sara K. Olson and Jeffrey D. Esko

Pteridines, Pages 556-560, S. Colette Daubner and Paul F. Fitzpatrick

P-Type Pumps: Copper Pump, Pages 561-564, Ilia Voskoboinik and James Camakaris

P-Type Pumps: H+/K+ Pump, Pages 565-570, Jai Moo Shin and George Sachs

P-Type Pumps: Na+/K+ Pump, Pages 571-576, Peter L. Jorgensen

P-Type Pumps: Plasma-Membrane H+ Pump, Pages 577-581, A. Brett Mason and Carolyn W. Slayman

Purple Bacteria: Electron Acceptors and Donors, Pages 582-585, Evaldas Katilius and Neal W. Woodbury

Purple Bacteria: Photosynthetic Reaction Centers, Pages 586-594, C. Roy D. Lancaster

Pyridoxal Phosphate, Pages 595-599, David E. Metzler

Pyrimidine Biosynthesis, Pages 600-605, Monika Loffler and Elke Zameitat

Pyruvate Carboxylation, Transamination, and Gluconeogenesis, Pages 606-610, Sarawut Jitrapakdee and John C. Wallace

Pyruvate Dehydrogenase, Pages 611-615, Sam A. Johnson and James G. McCormack

Pyruvate Kinase, Pages 616-619, Kosaku Uyeda

Quinones, Pages 621-627, Giorgio Lenaz and Maria Luisa Genova

Rab Family, Pages 629-634, Mary W. McCaffrey and Andrew J. Lindsay

Ran GTPase, Pages 635-639, Mary Shannon Moore

Ras Family, Pages 640-644, Lawrence A. Quilliam

Recombination: Heteroduplexand Mismatch Repair in vitro, Pages 645-648, Leroy Worth

Recombination: Strand Transferases, Pages 649-653, Floyd R. Bryant

Recombination-Dependent DNA Replication, Pages 654-659, Kenneth N. Kreuzer

recQ DNA Helicase Family in Genetic Stability, Pages 660-664, Mary A. Risinger and Joanna Groden

Regulated Intramembrane Proteolysis (Rip), Pages 665-670, Jin Ye, Michael S. Brown and Joseph L. Goldstein

Respiratory Chain and ATP Synthase, Pages 671-675, David G. Whitehouse and Anthony L. Moore

Respiratory Chain Complex I, Pages 676-680, Ulrich Brandt

Respiratory Chain Complex II and Succinate: Quinone Oxidoreductases, Pages 681-687, C. Roy D. Lancaster

Respiratory Chain Complex IV, Pages 688-694, Hartmut Michel

Respiratory Processes in Anoxygenic and Oxygenic Phototrophs, Pages 695-699, Roberto Borghese and Davide Zannoni

Retinoblastoma Protein (pRB), Pages 700-703, Nicholas Dyson and Maxim Frolov

Retinoic Acid Receptors, Pages 704-707, Martin Petkovich

Reverse Transcriptase and Retroviral Replication, Pages 708-713, Laura Tarrago-Litvak, Marie-Line Andreola and Simon Litvak

Rho GTPases and Actin Cytoskeleton Dynamics, Pages 714-718, Priam Villalonga and Anne J. Ridley

Ribosome Assembly, Pages 719-724, John L. Woolford

Ribosome Structure, Pages 725-732, Brian T. Wimberly

Ribozyme Mechanisms, Pages 733-737, John Hsieh and Carol A. Fierke

Ribozyme Structural Elements: Group I Introns, Pages 738-742, Barbara L. Golden

Ribozyme Structural Elements: Hairpin Ribozyme, Pages 743-746, Adrian R. Ferre-D'Amare

Ribozymes and Evolution, Pages 747-752, Niles Lehman

RNA Editing, Pages 753-758, Charles E. Samuel

RNA Polymerase I and RNA Polymerase III in Eukaryotes, Pages 759-762, Robert J. White

RNA Polymerase II and Basal Transcription Factors in Eukaryotes, Pages 763-765, Joan Weliky Conaway and Ronald C. Conaway

RNA Polymerase II Elongation Control in Eukaryotes, Pages 766-769, David H. Price

RNA Polymerase II Structure in Eukaryotes, Pages 770-774, Patrick Cramer

RNA Polymerase Reaction in Bacteria, Pages 775-780, Arkady A. Mustaev and Alexander D. Goldfarb

RNA Polymerase Structure, Bacterial, Pages 781-784, Michael Anikin, Dmitri Temiakov and William T. McAllister

## Volume 4

Secondary Structure in Protein Analysis, Pages 1-6, George D. Rose

Secretases, Pages 7-10, Robert L. Heinrikson

Secretory Pathway, Pages 11-16, Karen J. Colley

Selenoprotein Synthesis, Pages 17-21, August Bock

Septins and Cytokinesis, Pages 22-26, Makoto Kinoshita and Christine M. Field

Serine/Threonine Phosphatases, Pages 27-32, Thomas S. Ingebritsen

Serotonin Receptor Signaling, Pages 33-37, Paul J. Gresch and Elaine Sanders-Bush

Siglecs, Pages 38-40, Ajit Varki

Sigma Factors, Pages 41-44, John D. Helmann

Sliding Clamps in DNA Replication: E. coli -Clamp and PCNA Structure, Pages 45-47, Eric R. Goedken and John Kuriyan

Small GTPases, Pages 48-54, Adam Shutes and Channing J. Der

Somatostatin Receptors, Pages 55-60, Agnes Schonbrunn

Spastic Paraplegia, Pages 61-66, Elena Irene Rugarli and Andrea Ballabio

Spectrophotometric Assays, Pages 67-75, Britton Chance

Sphingolipid Biosynthesis, Pages 76-81, Martina Leipelt and Alfred H. Merrill

Sphingolipid Catabolism, Pages 82-87, Akira Abe and James A. Shayman

Spliceosome, Pages 88-92, Timothy W. Nilsen

Src Family of Protein Tyrosine Kinases, Pages 93-98, Jonathan A. Cooper

Starvation, Pages 99-110, Oliver E. Owen and Richard W. Hanson

Steroid/Thyroid Hormone Receptors, Pages 111-116, Ramesh Narayanan and Nancy L. Weigel

Store-Operated Membrane Channels: Calcium, Pages 117-122, Indu S. Ambudkar

Substrate Binding, Catalysis, and Product Release , Pages 123-126, W. Wallace Cleland

Sugar Nucleotide Transporters, Pages 127-129, Carlos B. Hirschberg

SUMO Modification, Pages 130-134, Frauke Melchior and Andrea Pichler

Superoxide Dismutase, Pages 135-138, Irwin Fridovich

Syk Family of Protein Tyrosine Kinases, Pages 139-145, Andrew C. Chan

T7 RNA Polymerase, Pages 147-151, Rui Sousa

Tachykinin/Substance P Receptors, Pages 152-157, Mark D. Richardson and Madan M. Kwatra

Taste Receptors, Pages 158-161, John D. Boughter, Jr. and Steven D. Munger

T-Cell Antigen Receptor, Pages 162-168, Andrea L. Szymczak and Dario A. A. Vignali

Tec/Btk Family Tyrosine Kinases, Pages 169-173, Shuling Guo and Owen N. Witte

Telomeres: Maintenance and Replication, Pages 174-179, Alessandro Bianchi and David Shore

Thyroid-Stimulating Hormone/Luteinizing Hormone/Follicle-Stimulating Hormone Receptors, Pages 180-186, Deborah L. Segaloff, Dario Mizrachi and Mario Ascoli

Tight Junctions, Pages 187-189, Shoichiro Tsukita

Title, Pages, Authors

Toll-Like Receptors, Pages 190-194, Himanshu Kumar, Kiyoshi Takeda and Shizuo Akira

Transcription Termination, Pages 195-199, Thomas J. Santangelo and Jeffrey W. Roberts

Transcriptional Silencing, Pages 200-203, Ann Sutton and Rolf Sternglanz

Transcription-Coupled DNA Repair, Overview, Pages 204-208, Isabel Mellon

Transforming Growth Factor-β Receptor Superfamily, Pages 209-213, Mark de Caestecker

Translation Elongation in Bacteria, Pages 214-223, Oliver Vesper and Knud H. Nierhaus

Translation Elongation in Eukaryotes, Pages 224-229, William C. Merrick and Anton A. Komar

Translation Initiation in Bacteria: Factors and Mechanisms, Pages 230-236, Cynthia L. Pon and Claudio O. Gualerzi

Translation Initiation in Eukaryotes: Factors and Mechanisms, Pages 237-241, Tatyana V. Pestova and Christopher U. T. Hellen

Translation Termination and Ribosome Recycling, Pages 242-246, Nadja Koloteva-Levin and Mick F. Tuite

Translesion DNA Polymerases, Eukaryotic, Pages 247-250, Alexandra Vaisman and Roger Woodgate

Trehalose Metabolism, Pages 251-255, Alan D. Elbein

Tricarboxylic Acid Cycle, Pages 256-262, Richard L. Veech

tRNA Synthetases, Pages 263-266, Karla L. Ewalt and Paul Schimmel

trp Operon and Attenuation, Pages 267-271, Paul Gollnick

Tubulin and its Isoforms, Pages 272-276, Eva Nogales

Tumor Necrosis Factor Receptors, Pages 277-283, Karen G. Potter and Carl F. Ware

Two-Dimensional Gel Electrophoresis, Pages 284-289, Gerhard Schmid, Denis Hochstrasser and Jean-Charles Sanchez

Two-Hybrid Protein-Protein Interactions, Pages 290-293, Ilya Serebriiskii and Erica A. Golemis

Tyrosine Sulfation, Pages 294-297, Denis Corbeil and Wieland B. Huttner

Ubiquitin System, Pages 299-303, Aaron Ciechanover and Michael H. Glickman

Ubiquitin-Like Proteins, Pages 304-307, Edward T. H. Yeh

UmuC, D Lesion Bypass DNA Polymerase V, Pages 308-312, Zvi Livneh

Uncoupling Proteins, Pages 313-318, Daniel Ricquier and Frederic Bouillaud

Unfolded Protein Responses, Pages 319-325, David Ron

Urea Cycle, Inborn Defects of, Pages 326-330, Marsha K. Fearing and Vivian E. Shih

Vacuoles, Pages 331-336, Christopher J. Stefan and Scott D. Emr

Vascular Endothelial Growth Factor Receptors, Pages 337-342, Kenneth A. Thomas

Vasopressin/Oxytocin Receptor Family, Pages 343-348, Michael J. Brownstein

V-ATPases, Pages 349-353, Michael Forgac

Vitamin A (Retinoids), Pages 354-359, Joseph L. Napoli

Vitamin B12 and B12-Proteins, Pages 360-366, Bernhard Krautler

Vitamin C, Pages 367-371, Robert B. Rucker and Francene Steinberg

Vitamin D, Pages 372-377, Hector F. DeLuca and Margaret Clagett-Dame

Vitamin D Receptor, Pages 378-383, Diane R. Dowd and Paul N. MacDonald

Vitamin E, Pages 384-388, Ute C. Obermuller-Jevic and Lester Packer

Vitamin K: Biochemistry, Metabolism, and Nutritional Aspects, Pages 389-393, J. W. Suttie

Vitamin K: Blood Coagulation and Use in Therapy, Pages 394-398, Matthew D. Stone and Gary L. Nelsestuen

Voltage-Dependent K+ Channels, Pages 399-404, Ramon Latorre and Francisco J. Morera

Voltage-Sensitive Ca2+ Channels, Pages 405-408, Harald Reuter

Voltage-Sensitive Na+ Channels, Pages 409-415, William J. Brammar

Von Hippel-Lindau (VHL) Protein, Pages 416-418, Ronald C. Conaway and Joan Weliky Conaway

XPV DNA Polymerase and Ultraviolet Damage Bypass, Pages 419-421, Alan R. Lehmann

X-Ray Determination of 3-D Structure in Proteins, Pages 422-428, Martha L. Ludwig

Yeast GAL1-GAL10 System, Pages 429-433, Dennis Lohr and Ralph Bash

Zinc Fingers, Pages 435-439, Mark Isalan

**Lipids, Carbohydrates, Membranes and Membrane Proteins**

Carbohydrate Chains: Enzymatic and Chemical Synthesis; Vol.1 - Pages 307-313, Thomas J. Tolbert and Chi-Huey Wong

Cell–Matrix Interactions; Vol.1 - Pages 362-366, Janet A. Askari and Martin J. Humphries

Detergent Properties; Vol.1 - Pages 577-581, Darrell R. McCaslin

Endocytosis; Vol.2 - Pages 16-19, Julie G. Donaldson

Flippases; Vol.2 - Pages 123-127, Charles J. Waechter

Galectins; Vol.2 - Pages 171-174, R. Colin Hughes

GlcNAc Biosynthesis and Function, O-Linked; Vol.2 - Pages 189-192, Kaoru Sakabe and Gerald W. Hart

Glycation; Vol.2 - Pages 229-236, Suzanne R. Thorpe and John W. Baynes

Glycolipid-Dependent Adhesion Processes; Vol.2 - Pages 261-265, Senitiroh Hakomori

Glycoprotein Folding and Processing Reactions; Vol.2 - Pages 272-276, Armando J. Parodi

Glycoprotein-Mediated Cell Interactions, O-Linked; Vol.2 - Pages 277-282, Robert S. Haltiwanger

Glycoproteins, N-Linked; Vol.2 - Pages 283-292, Mark A. Lehrman

Glycoproteins, Plant; Vol.2 - Pages 293-296, Carolyn J. Schultz

Glycosylation in Cystic Fibrosis; Vol.2 - Pages 297-301, Andrew D. Rhim, Thomas F. Scanlin and Mary Catherine Glick

Glycosylation, Congenital Disorders of; Vol.2 - Pages 302-307, Hudson H. Freeze

Glycosylphosphatidylinositol (GPI) Anchors; Vol.2 - Pages 308-311, Anant K. Menon

Ion Channel Protein Superfamily; Vol.2 - Pages 473-477, William A. Catterall

Lectins; Vol.2 - Pages 535-540, Nathan Sharon and Halina Lis

Lipases; Vol.2 - Pages 571-575, Howard L. Brockman

Lipid Bilayer Structure; Vol.2 - Pages 576-579, Erwin London

Lipid Rafts; Vol.2 - Pages 584-587, Deborah A. Brown

Lipoproteins, HDL/LDL; Vol.2 - Pages 588-593, Fayanne E. Thorngate and David L. Williams

MDR Membrane Proteins; Vol.2 - Pages 605-609, Nathan C. Rockwell

Membrane Fusion; Vol.2 - Pages 621-626, Joshua Zimmerberg and Leonid V. Chernomordik

Mucin Family of Glycoproteins; Vol.2 - Pages 758-764, Juan Perez-Vilar and Robert L. Hill

Mucins in Embryo Implantation; Vol.2 - Pages 765-769, Daniel D. Carson

Neoglycoproteins; Vol.3 - Pages 11-15, Y. C. Lee and Reiko T. Lee

N-Linked Glycan Processing Glucosidases and Mannosidases; Vol.3 - Pages 46-49, Linda O. Tremblay and Annette Herscovics

Oligosaccharide Chains: Free, N-Linked, O-Linked; Vol.3 - Pages 161-164, Tadashi Suzuki

Phospholipid Metabolism in Mammals; Vol.3 - Pages 314-320, Claudia Kent

Phospholipid Synthesis in Yeast; Vol.3 - Pages 321-325, Gil-Soo Han and George M. Carman

Prostaglandins and Leukotrienes; Vol.3 - Pages 452-456, William L. Smith and Robert C. Murphy

Protein Glycosylation Inhibitors; Vol.3 - Pages 500-503, Alan D. Elbein

Proteoglycans; Vol.3 - Pages 549-555, Sara K. Olson and Jeffrey D. Esko

Secretory Pathway; Vol.4 - Pages 11-16, Karen J. Colley

Siglecs; Vol.4 - Pages 38-40, Ajit Varki

Sphingolipid Biosynthesis; Vol.4 - Pages 76-81, Martina Leipelt and Alfred H. Merrill, Jr.

Sphingolipid Catabolism; Vol.4 - Pages 82-87, Akira Abe and James A. Shayman

Sugar Nucleotide Transporters; Vol.4 - Pages 127-129, Carlos B. Hirschberg

Trehalose Metabolism; Vol.4 - Pages 251-255, Alan D. Elbein

**Metabolism, Vitamins and Hormones**

Amino Acid Metabolism; Vol.1 - Pages 90-95, Luc Cynober

Anaplerosis; Vol.1 - Pages 105-110, Raymond R. Russell, III and Heinrich Taegtmeyer

Bile Salts and their Metabolism; Vol.1 - Pages 159-163, Ulrich Beuers and Thomas Pusl

Biliary Cirrhosis, Primary; Vol.1 - Pages 164-169, Marshall M. Kaplan

Branched-Chain -Ketoacids; Vol.1 - Pages 186-191, David T. Chuang

Carnitine and -Oxidation; Vol.1 - Pages 314-318, Janos Kerner and Charles L. Hoppel

Cholesterol Synthesis; Vol.1 - Pages 451-455, Peter A. Edwards

Diabetes; Vol.1 - Pages 582-592, David W. Cooke

Fat Mobilization: Perilipin and Hormone-Sensitive Lipase; Vol.2 - Pages 85-89, Constantine Londos and Alan R. Kimmel

Fatty Acid Oxidation; Vol.2 - Pages 90-94, Horst Schulz

Fatty Acid Synthesis and its Regulation; Vol.2 - Pages 99-103, Steven D. Clarke and Manabu T. Nakamura

Gluconeogenesis; Vol.2 - Pages 197-203, Richard W. Hanson and Oliver E. Owen

Glucose/Sugar Transport in Bacteria; Vol.2 - Pages 204-207, Lan Guan and H. Ronald Kaback

Glucose/Sugar Transport in Mammals; Vol.2 - Pages 208-212, Silvia Mora and Jeffrey Pessin

Glycogen Metabolism; Vol.2 - Pages 244-248, Peter J. Roach

Glycogen Storage Diseases; Vol.2 - Pages 249-254, George H. Sack, Jr.

Glycolysis, Overview; Vol.2 - Pages 266-271, Robert A. Harris

Hexokinases/Glucokinases; Vol.2 - Pages 372-377, Emile Van Schaftingen

Insulin- and Glucagon-Secreting Cells of the Pancreas; Vol.2 - Pages 430-435, Franz M. Matschinsky

Ketogenesis; Vol.2 - Pages 505-507, Janos Kerner and Charles L. Hoppel

Ornithine Cycle; Vol.3 - Pages 172-177, Malcolm Watford

Pentose Phosphate (Hexose Mono Phosphate) Pathway; Vol.3 - Pages 211-215, Bernard R. Landau

Pentose Phosphate Pathway, History of; Vol.3 - Pages 216-225, John F. Williams

Phosphofructokinase-2/Fructose Bisphosphatase-2; Vol.3 - Pages 277-280, Daniel M. Raben

Photosynthesis; Vol.3 - Pages 330-335, Richard C. Leegood

Photosynthetic Carbon Dioxide Fixation; Vol.3 - Pages 336-341, Matthew J. Paul

Porphyrin Metabolism; Vol.3 - Pages 415-419, Mark Shepherd and Harry A. Dailey

Propionyl CoA–Succinyl CoA Pathway; Vol.3 - Pages 447-451, Wayne A. Fenton

Pyruvate Carboxylation, Transamination, and Gluconeogenesis; Vol.3 - Pages 606-610, Sarawut Jitrapakdee and John C. Wallace

Pyruvate Dehydrogenase; Vol.3 - Pages 611-615, Sam A. Johnson and James G. McCormack

Pyruvate Kinase; Vol.3 - Pages 616-619, Kosaku Uyeda

Starvation; Vol.4 - Pages 99-110, Oliver E. Owen and Richard W. Hanson

Tricarboxylic Acid Cycle; Vol.4 - Pages 256-262, Richard L. Veech

Urea Cycle, Inborn Defects of; Vol.4 - Pages 326-330, Marsha K. Fearing and Vivian E. Shih

Vitamin A (Retinoids); Vol.4 - Pages 354-359, Joseph L. Napoli

Vitamin B12 and B12-Proteins; Vol.4 - Pages 360-366, Bernhard Krautler

Vitamin C; Vol.4 - Pages 367-371, Robert B. Rucker and Francene Steinberg

Vitamin D; Vol.4 - Pages 372-377, Hector F. DeLuca and Margaret Clagett-Dame

Vitamin E; Vol.4 - Pages 384-388, Ute C. Obermuller-Jevic and Lester Packer

Vitamin K: Biochemistry, Metabolism, and Nutritional Aspects; Vol.4 - Pages 389-393, J. W. Suttie

Vitamin K: Blood Coagulation and Use in Therapy; Vol.4 - Pages 394-398, Matthew D. Stone and Gary L. Nelsestuen

## Cell Architecture and Function

Actin Assembly/Disassembly; Vol.1 - Pages 12-18, Henry N. Higgs

Actin-Capping and -Severing Proteins; Vol.1 - Pages 19-26, Sankar Maiti and James R. Bamburg

Actin-Related Proteins; Vol.1 - Pages 27-33, R. Dyche Mullins

Autophagy in Fungi and Mammals; Vol.1 - Pages 138-143, Daniel J. Klionsky and Ju Guan

Bax and Bcl2 Cell Death Enhancers and Inhibitors; Vol.1 - Pages 152-154, David L. Vaux

Cadherin-Mediated Cell–Cell Adhesion; Vol.1 - Pages 205-211, Frauke Drees and W. James Nelson

Caspases and Cell Death; Vol.1 - Pages 319-327, Don W. Nicholson, Pierluigi Nicotera and Gerry Melino

Cell Cycle Controls in G1 and G0; Vol.1 - Pages 328-331, WengeShi and Steven F. Dowdy

Cell Cycle: Control of Entry and Progression Through S Phase; Vol.1 - Pages 332-337, Susan L. Forsburg

Cell Cycle: DNA Damage Checkpoints; Vol.1 - Pages 338-344, Jean Y. J. Wang

Cell Cycle: Mitotic Checkpoint; Vol.1 - Pages 345-351, Tim J. Yen

Cell Migration; Vol.1 - Pages 356-361, J. Victor Small and Emmanuel Vignal

Centromeres; Vol.1 - Pages 367-371, Beth A. Sullivan

Centrosomes and Microtubule Nucleation; Vol.1 - Pages 372-376, Reiko Nakajima, Ming-Ying Tsai and Yixian Zheng

Chaperones for Metalloproteins; Vol.1 - Pages 383-386, Valeria C. Culotta and Edward Luk

Chaperones, Molecular; Vol.1 - Pages 387-392, Sue Wickner and Joel R. Hoskins

Chaperonins; Vol.1 - Pages 393-398, Arthur L. Horwich, Wayne A. Fenton and George W. Farr

Chromosome Organization and Structure, Overview; Vol.1 - Pages 469-474, Elena Gracheva and Sarah C. R. Elgin

Cytokinesis; Vol.1 - Pages 556-561, Masanori Mishima and Michael Glotzer

Desmosomes and Hemidesmosomes; Vol.1 - Pages 569-576, Rachel L. Dusek, Jonathan C. R. Jones and Kathleen J. Green

Dynactin; Vol.1 - Pages 823-826, Trina A. Schroer

Dynein; Vol.1 - Pages 827-831, K. Kevin Pfister

Endoplasmic Reticulum-Associated Protein Degradation; Vol.2 - Pages 20-23, Maurizio Molinari

Focal Adhesions; Vol.2 - Pages 128-133, Eli Zamir and Benjamin Geiger

Golgi Complex; Vol.2 - Pages 312-315, Mark Stamnes

Heat/Stress Responses; Vol.2 - Pages 343-347, Davis T. W. Ng

Inositol Lipid 3-Phosphatases; Vol.2 - Pages 421-426, Gregory S. Taylor and Jack E. Dixon

Intermediate Filament Linker Proteins: Plectin and BPAG1; Vol.2 - Pages 452-457, Peter Fuchs and Gerhard Wiche

Intermediate Filaments; Vol.2 - Pages 458-464, Kelsie M. Bernot and Pierre A. Coulombe

Keratins and the Skin; Vol.2 - Pages 497-504, Pierre A. Coulombe and Kelsie M. Bernot

Kinesin Superfamily Proteins; Vol.2 - Pages 508-516, Nobutaka Hirokawa and Reiko Takemura

Kinesins as Microtubule Disassembly Enzymes; Vol.2 - Pages 517-521, Susan L. Kline-Smith and Arshad Desai

Meiosis; Vol.2 - Pages 610-616, Neil Hunter

Metalloproteinases, Matrix; Vol.2 - Pages 657-665, Hideaki Nagase and Gillian Murphy

Microtubule-Associated Proteins; Vol.2 - Pages 676-682, Nobutaka Hirokawa and Reiko Takemura

Mitosis; Vol.2 - Pages 743-747, Patricia Wadsworth and Nasser M. Rusan

Myosin Motors; Vol.2 - Pages 778-781, Roy E. Larson

N-End Rule; Vol.3 - Pages 6-10, Alexander Varshavsky

Neuronal Intermediate Filaments; Vol.3 - Pages 21-25, Ronald K. H. Liem

Nuclear Compartmentalization; Vol.3 - Pages 87-91, Kelly P. Smith and Jeanne B. Lawrence

Nuclear Envelope and Lamins; Vol.3 - Pages 92-95, Bryce M. Paschal

Nuclear Pores and Nuclear Import/Export; Vol.3 - Pages 109-114, Anita H. Corbett

Peroxisomes; Vol.3 - Pages 246-250, Suresh Subramani

26S Proteasome, Structure and Function; Vol.3 - Pages 469-473, Peter Zwickl and Wolfgang Baumeister

Protein Glycosylation, Overview; Vol.3 - Pages 504-509, Natasha E. Zachara and Gerald W. Hart

Rho GTPases and Actin Cytoskeleton Dynamics; Vol.3 - Pages 714-718, Priam Villalonga and Anne J. Ridley

Septins and Cytokinesis; Vol.4 - Pages 22-26, Makoto Kinoshita and Christine M. Field

SUMO Modification; Vol.4 - Pages 130-134, Frauke Melchior and Andrea Pichler

Tight Junctions; Vol.4 - Pages 187-189, Shoichiro Tsukita

Transcriptional Silencing; Vol.4 - Pages 200-203, Ann Sutton and Rolf Sternglanz

Tubulin and its Isoforms; Vol.4 - Pages 272-276, Eva Nogales

Unfolded Protein Responses; Vol.4 - Pages 319-325, David Ron

Vacuoles; Vol.4 - Pages 331-336, Christopher J. Stefan and Scott D. Emr

**Protein/Enzyme Structure  Function and Degradation**

Allosteric Regulation; Vol.1 - Pages 68-73, Barry S. Cooperman

Aminopeptidases; Vol.1 - Pages 96-98, Ralph A. Bradshaw

Amyloid; Vol.1 - Pages 99-104, Ronald Wetzel

Aspartic Proteases; Vol.1 - Pages 123-127, Ben M. Dunn

B12-Containing Enzymes; Vol.1 - Pages 145-151, Vahe Bandarian and Rowena G. Matthews

Biotin; Vol.1 - Pages 174-178, Steven W. Polyak and Anne Chapman-Smith

Biotinylation of Proteins; Vol.1 - Pages 179-181, Ronald A. Kohanski

Calpain; Vol.1 - Pages 300-306, Hiroyuki Sorimachi and Yasuko Ono

Chemiluminescence and Bioluminescence; Vol.1 - Pages 399-404, Thomas O. Baldwin

Coenzyme A; Vol.1 - Pages 475-477, M. Daniel Lane

Collagenases; Vol.1 - Pages 478-481, Kenn Holmbeck and Henning Birkedal-Hansen

Collagens; Vol.1 - Pages 482-487, Darwin J. Prockop

Cysteine Proteases; Vol.1 - Pages 516-520, David J. Buttle and John S. Mort

Disulfide Bond Formation; Vol.1 - Pages 598-602, Hiram F. Gilbert

Elastin; Vol.2 - Pages 10-12, Judith Ann Foster

Enzyme Inhibitors; Vol.2 - Pages 31-37, Vern L. Schramm

Enzyme Kinetics; Vol.2 - Pages 38-44, Irwin H. Segel

Enzyme Reaction Mechanisms: Stereochemistry; Vol.2 - Pages 45-50, Ming-Daw Tsai, Li Zhao and Brandon J. Lamarche

Flavins; Vol.2 - Pages 118-122, Barrie Entsch and David P. Ballou

Heme Proteins; Vol.2 - Pages 354-361, Johannes Everse

HIV Protease; Vol.2 - Pages 384-387, Ben M. Dunn

Kinetic Isotope Effects; Vol.2 - Pages 522-527, Justine P. Roth and Judith P. Klinman

Lipid Modification of Proteins: Targeting to Membranes; Vol.2 - Pages 580-583, Marilyn D. Resh

Low Barrier Hydrogen Bonds; Vol.2 - Pages 594-598, Perry A. Frey

Metalloproteases; Vol.2 - Pages 652-656, David S. Auld

Peptide Amidation; Vol.3 - Pages 226-230, Mark J. Niciu, Richard E. Mains and Betty A. Eipper

Phage Display for Protein Binding; Vol.3 - Pages 251-255, Henry B. Lowman

Prions, Overview; Vol.3 - Pages 437-440, Cedric Govaerts and Fred E. Cohen

Proteases in Blood Clotting; Vol.3 - Pages 457-463, John D. Kulman and Earl W. Davie

Proteasomes, Overview; Vol.3 - Pages 464-468, Martin Rechsteiner

Protein Carboxyl Esterification; Vol.3 - Pages 474-477, Shilpa G. Lalchandani and Jeffry B. Stock

Protein Degradation; Vol.3 - Pages 484-492, Tomo ari and Alfred L. Goldberg

Protein Folding and Assembly; Vol.3 - Pages 493-499, David P. Goldenberg

Protein N-Myristoylation; Vol.3 - Pages 527-531, Erica Dutil Sonnenburg and Jeffrey I. Gordon

Protein Palmitoylation; Vol.3 - Pages 532-535, Robert J. Deschenes

Pteridines; Vol.3 - Pages 556-560, S. Colette Daubner and Paul F. Fitzpatrick

Pyridoxal Phosphate; Vol.3 - Pages 595-599, David E. Metzler

Regulated Intramembrane Proteolysis (Rip); Vol.3 - Pages 665-670, Jin Ye, Michael S. Brown and Joseph L. Goldstein

Secretases; Vol.4 - Pages 7-10, Robert L. Heinrikson

Selenoprotein Synthesis; Vol.4 - Pages 17-21, August Bock

Substrate Binding, Catalysis, and Product Release; Vol.4 - Pages 123-126, W. Wallace Cleland

Two-Hybrid Protein–Protein Interactions; Vol.4 - Pages 290-293, Ilya Serebriiskii and Erica A. Golemis

Tyrosine Sulfation; Vol.4 - Pages 294-297, Denis Corbeil and Wieland B. Huttner

Ubiquitin System; Vol.4 - Pages 299-303, Aaron Ciechanover and Michael H. Glickman

Ubiquitin-Like Proteins; Vol.4 - Pages 304-307, Edward T. H. Yeh

Zinc Fingers; Vol.4 - Pages 435-439, Mark Isalan

**Bioenergetics**

ABC Transporters; Vol.1 - Pages 1-5, Andre Goffeau, Benoit De Hertogh and Philippe V. Baret

Amine Oxidases; Vol.1 - Pages 85-89, Giovanni Floris and Alessandro Finazzi Agro

ATP Synthesis in Plant Mitochondria: Substrates, Inhibitors, Uncouplers; Vol.1 - Pages 128-132, Kathleen L. Soole and R. Ian Menz

ATP Synthesis: Mitochondrial Cyanide-Resistant Terminal Oxidases; Vol.1 - Pages 133-137, James N. Siedow

Bioenergetics: General Definition of Principles; Vol.1 - Pages 170-173, David G. Nicholls

Calcium Buffering Proteins: Calbindin; Vol.1 - Pages 221-225, Willi Hunziker and Igor Bendik

Calcium Buffering Proteins: ER Luminal Proteins; Vol.1 - Pages 226-230, Jody Groenendyk and Marek Michalak

Calcium Oscillations; Vol.1 - Pages 231-234, Marisa Brini

Calcium Sensing Receptor; Vol.1 - Pages 235-240, Jacob Tfelt-Hansen and Edward M. Brown

Calcium Signaling: Calmodulin-Dependent Phosphatase; Vol.1 - Pages 241-245, Claude Klee, Hao Ren and Shipeng Li

Calcium Signaling: Cell Cycle; Vol.1 - Pages 246-249, Luigia Santella

Calcium Signaling: Motility (Actomyosin–Troponin System); Vol.1 - Pages 250-255, Takeyuki Wakabayashi and Setsuro Ebashi

Calcium Signaling: NO Synthase; Vol.1 - Pages 256-260, Zhi-Qiang Wang and Dennis J. Stuehr

Calcium Transport in Mitochondria; Vol.1 - Pages 261-266, Rosario Rizzuto and Marisa Brini

Calcium Waves; Vol.1 - Pages 267-269, Lionel F. Jaffe

Calcium, Biological Fitness of; Vol.1 - Pages 270-273, Robert J. P. Williams

Calcium/Calmodulin-Dependent Protein Kinase II; Vol.1 - Pages 274-280, Andy Hudmon and Howard Schulman

Calcium-Binding Proteins: Cytosolic (Annexins, Gelsolins, C2-Domain Proteins); Vol.1 - Pages 287-293, Joachim Krebs

Calcium-Modulated Proteins (EF-Hand); Vol.1 - Pages 294-299, Robert H. Kretsinger

Cell Death by Apoptosis and Necrosis; Vol.1 - Pages 352-355, Pierluigi Nicotera

Chemiosmotic Theory; Vol.1 - Pages 405-412, Keith D. Garlid

Chemolithotrophy; Vol.1 - Pages 419-424, Alan B. Hooper

Chlorophylls and Carotenoids; Vol.1 - Pages 430-437, Hugo Scheer

Chloroplast Redox Poise and Signaling; Vol.1 - Pages 438-445, John F. Allen

Chloroplasts; Vol.1 - Pages 446-450, Nicoletta Rascio

Cytochrome b6f Complex; Vol.1 - Pages 521-527, Gunter A. Hauska and Thomas Schodl

Cytochrome bc1 Complex (Respiratory Chain Complex III); Vol.1 - Pages 528-534, Bernard L. Trumpower

Cytochrome c; Vol.1 - Pages 535-538, Hans Tuppy and Gunther Kreil

Cytochrome Oxidases, Bacterial; Vol.1 - Pages 539-543, Peter Brzezinski and Pia Adelroth

Cytochrome P-450; Vol.1 - Pages 544-549, Rita Bernhardt

Energy Transduction in Anaerobic Prokaryotes; Vol.2 - Pages 24-30, Gottfried Unden

ER/SR Calcium Pump: Function; Vol.2 - Pages 56-60, Giuseppe Inesi

ER/SR Calcium Pump: Structure; Vol.2 - Pages 61-65, Chikashi Toyoshima and Yuji Sugita

F1–F0 ATP Synthase; Vol.2 - Pages 73-79, Donata Branca

Ferredoxin; Vol.2 - Pages 104-106, Giuliana Zanetti and Vittorio Pandini

Ferredoxin-NADP+ Reductase; Vol.2 - Pages 107-111, Giuliana Zanetti and Alessandro Aliverti

Free Radicals, Sources and Targets of: Mitochondria; Vol.2 - Pages 134-142, Alberto Boveris and Enrique Cadenas

Friedreich's Ataxia; Vol.2 - Pages 143-145, Paul E. Hart and Anthony H. V. Schapira

Giant Mitochondria (Megamitochondria); Vol.2 - Pages 186-188, Bernard Tandler and Charles L. Hoppel

Glutathione Peroxidases; Vol.2 - Pages 224-228, Fulvio Ursini and Matilde Maiorino

Green Bacteria: Secondary Electron Donor (Cytochromes); Vol.2 - Pages 321-324, Hirozo Oh-oka and Robert E. Blankenship

Green Bacteria: The Light-Harvesting Chlorosome; Vol.2 - Pages 325-330, John M. Olson

Green Sulfur Bacteria: Reaction Center and Electron Transport; Vol.2 - Pages 331-336, Gunter A. Hauska and Thomas Schodl

Heme Synthesis; Vol.2 - Pages 362-366, Gloria C. Ferreira

Intracellular Calcium Channels: cADPR-Modulated (Ryanodine Receptors); Vol.2 - Pages 465-468, Antony Galione

Intracellular Calcium Channels: NAADP+-Modulated; Vol.2 - Pages 469-472, Armando A. Genazzani and Marcella Debidda

IP3 Receptors; Vol.2 - Pages 478-481, Colin W. TaylorEdward Morris and Paula da Fonseca

Iron–Sulfur Proteins; Vol.2 - Pages 482-489, Helmut Beinert, Jacques Meyer and Roland Lill

Ligand-Operated Membrane Channels: Calcium (Glutamate); Vol.2 - Pages 551-561, Elias K. Michaelis

Ligand-Operated Membrane Channels: GABA; Vol.2 - Pages 562-566, F. Minier and Erwin Sigel

Light-Harvesting Complex (LHC) I and II: Pigments and Proteins; Vol.2 - Pages 567-570, Stefan Jansson

Luft's Disease; Vol.2 - Pages 599-601, Salvatore DiMauro

Membrane Transport, General Concepts; Vol.2 - Pages 627-630, Stanley G. Schultz

Membrane Transporters:Na+/Ca2+ Exchangers; Vol.2 - Pages 631-636, Jonathan Lytton

Membrane-Associated Energy Transduction in Bacteria and Archaea; Vol.2 - Pages 637-645, Gunter Schafer

Metabolite Channeling: Creatine Kinase Microcompartments; Vol.2 - Pages 646-651, Uwe Schlattner and Theo Wallimann

Mitochondrial Auto-Antibodies; Vol.2 - Pages 683-688, Harold Baum

Mitochondrial Channels; Vol.2 - Pages 689-692, M. Catia Sorgato and Alessandro Bertoli

Mitochondrial DNA; Vol.2 - Pages 693-696, Gottfried Schatz

Mitochondrial Genes and their Expression: Yeast; Vol.2 - Pages 697-702, Piotr P. Slonimski and Giovanna Carignani

Mitochondrial Genome, Evolution; Vol.2 - Pages 703-708, B. Franz Lang, Dennis V. Lavrov and Gertraud Burger

Mitochondrial Genome, Overview; Vol.2 - Pages 709-715, Douglas C. Wallace

Mitochondrial Inheritance; Vol.2 - Pages 716-719, Eric A. Shoubridge

Mitochondrial Membranes, Structural Organization; Vol.2 - Pages 720-724, Carmen A. Mannella

Mitochondrial Metabolite Transporter Family; Vol.2 - Pages 725-732, Ferdinando Palmieri and Martin Klingenberg

Mitochondrial Outer Membrane and the VDAC Channel; Vol.2 - Pages 733-736, Marco Colombini

Neuronal Calcium Signal; Vol.3 - Pages 16-20, Hilmar Bading

Nicotinamide Nucleotide Transhydrogenase; Vol.3 - Pages 50-56, Jan Rydstrom

Nuclear Genes in Mitochondrial Function and Biogenesis; Vol.3 - Pages 100-104, Alexander Tzagoloff and Carol L. Dieckmann

Oncocytes; Vol.3 - Pages 165-166, Bernard Tandler and Charles L. Hoppel

Oxygenases; Vol.3 - Pages 178-182, Osamu Hayaishi

Periplasmic Electron Transport Systems in Bacteria; Vol.3 - Pages 231-238, David J. Richardson, Gary Sawers and Rob J. M. Van Spanning

Phosphatidylinositol-3-Phosphate; Vol.3 - Pages 272-276, Joseph V. Virbasius and Michael P. Czech

Photosystem I, Structure and Function; Vol.3 - Pages 342-347, Petra Fromme

Photosystem I: FX, FA, and FB Iron–Sulfur Clusters; Vol.3 - Pages 348-356, Mikhail L. Antonkine and John H. Golbeck

Photosystem II Light Harvesting System: Dynamic Behavior; Vol.3 - Pages 357-362, Peter Horton and Alexander Ruban

Photosystem II: Assembly and Turnover of the D1 Protein; Vol.3 - Pages 363-366, Eira Kanervo and Eva-Mari Aro

Photosystem II: Protein Components; Vol.3 - Pages 367-374, James Barber

Photosystem II: Water Oxidation, Overview; Vol.3 - Pages 375-380, Fabrice Rappaport and Pierre Joliot

Plasma-Membrane Calcium Pump: Structure and Function; Vol.3 - Pages 385-389, Emanuel E. Strehler

Plastocyanin; Vol.3 - Pages 390-393, Elizabeth L. Gross

Protein Import into Mitochondria; Vol.3 - Pages 510-515, Johannes M. Herrmann and Walter Neupert

P-Type Pumps: Copper Pump; Vol.3 - Pages 561-564, Ilia Voskoboinik and James Camakaris

P-Type Pumps: H+/K+ Pump; Vol.3 - Pages 565-570, Jai Moo Shin and George Sachs

P-Type Pumps: Na+/K+ Pump; Vol.3 - Pages 571-576, Peter L. Jorgensen

P-Type Pumps: Plasma-Membrane H+ Pump; Vol.3 - Pages 577-581, A. Brett Mason and Carolyn W. Slayman

Purple Bacteria: Electron Acceptors and Donors; Vol.3 - Pages 582-585, Evaldas Katilius and Neal W. Woodbury

Purple Bacteria: Photosynthetic Reaction Centers; Vol.3 - Pages 586-594, C. Roy D. Lancaster

Pyrimidine Biosynthesis; Vol.3 - Pages 600-605, Monika Loffler and Elke Zameitat

Quinones; Vol.3 - Pages 621-627, Giorgio Lenaz and Maria Luisa Genova

Respiratory Chain and ATP Synthase; Vol.3 - Pages 671-675, David G. Whitehouse and Anthony L. Moore

Respiratory Chain Complex I; Vol.3 - Pages 676-680, Ulrich Brandt

Respiratory Chain Complex II and Succinate: Quinone Oxidoreductases; Vol.3 - Pages 681-687, C. Roy D. Lancaster

Respiratory Chain Complex IV; Vol.3 - Pages 688-694, Hartmut Michel

Respiratory Processes in Anoxygenic and Oxygenic Phototrophs; Vol.3 - Pages 695-699, Roberto Borghese and Davide Zannoni

Spastic Paraplegia; Vol.4 - Pages 61-66, Elena Irene Rugarli and Andrea Ballabio

Store-Operated Membrane Channels: Calcium; Vol.4 - Pages 117-122, Indu S. Ambudkar

Superoxide Dismutase; Vol.4 - Pages 135-138, Irwin Fridovich

Uncoupling Proteins; Vol.4 - Pages 313-318, Daniel Ricquier and Frederic Bouillaud

V-ATPases; Vol.4 - Pages 349-353, Michael Forgac

Voltage-Dependent K+ Channels; Vol.4 - Pages 399-404, Ramon Latorre and Francisco J. Morera

Voltage-Sensitive Ca2+ Channels; Vol.4 - Pages 405-408, Harald Reuter

Voltage-Sensitive Na+ Channels; Vol.4 - Pages 409-415, William J. Brammar

**Molecular Biology**

Alternative Splicing: Regulation of Fibroblast Growth Factor Receptor (FGFR); Vol.1 - Pages 74-77, Mariano A. Garcia-Blanco

Alternative Splicing: Regulation of Sex Determination in Drosophila melanogaster; Vol.1 - Pages 78-84, Jill K. M. Penn, Patricia Graham and Paul Schedl

ara Operon; Vol.1 - Pages 116-119, Robert F. Schleif

Chromatin Remodeling; Vol.1 - Pages 456-463, Eric Kallin and Yi Zhang

Chromatin: Physical Organization; Vol.1 - Pages 464-468, Christopher L. Woodcock

DNA Base Excision Repair; Vol.1 - Pages 603-608, Hilde Nilsen and Tomas Lindahl

DNA Damage: Alkylation; Vol.1 - Pages 609-613, Anton B. Guliaev and B. Singer

DNA Glycosylases: Mechanisms; Vol.1 - Pages 614-617, Daniel J. Krosky and James T. Stivers

DNA Helicases: Dimeric Enzyme Action; Vol.1 - Pages 618-623, Timothy M. Lohman

DNA Helicases: HexamericEnzyme Action; Vol.1 - Pages 624-631, Smita S. Patel

DNA Ligases: Mechanism and Functions; Vol.1 - Pages 632-636, Alan E. Tomkinson and John B. Leppard

DNA Ligases: Structures; Vol.1 - Pages 637-643, C. Kiong Ho, Mark Odell and Dimitar B. Nikolov

DNA Methyltransferases, Bacterial; Vol.1 - Pages 644-651, Albert Jeltsch and Richard I. Gumport

DNA Methyltransferases, Structural Themes; Vol.1 - Pages 652-659, Sanjay Kumar

DNA Methyltransferases: Eubacterial GATC; Vol.1 - Pages 660-664, Martin G. Marinus

DNA Mismatch Repair and Homologous Recombination; Vol.1 - Pages 665-670, Ivan Matic and Miroslav Radman

DNA Mismatch Repair and the DNA Damage Response; Vol.1 - Pages 671-674, Guo-Min Li and Steven R. Presnell

DNA Mismatch Repair Defects and Cancer; Vol.1 - Pages 675-681, Richard D. Kolodner

DNA Mismatch Repair in Bacteria; Vol.1 - Pages 682-686, A-Lien Lu

DNA Mismatch Repair in Mammals; Vol.1 - Pages 687-690, James T. Drummond

DNA Mismatch Repair: E. coli Vsr and Eukaryotic G–T Systems; Vol.1 - Pages 691-693, Margaret Lieb

DNA Oxidation; Vol.1 - Pages 694-697, Arthur P. Grollman and Dmitry O. Zharkov

DNA Photolyase; Vol.1 - Pages 698-702, Carrie L. Partch and Aziz Sancar

DNA Polymerase α, Eukaryotic; Vol.1 - Pages 703-707, Teresa S. -F. Wang

DNA Polymerase β, Eukaryotic; Vol.1 - Pages 708-712, William A. Beard and Samuel H. Wilson

DNA Polymerase δ, Eukaryotic; Vol.1 - Pages 713-715, Antero G. So and Kathleen M. Downey

DNA Polymerase ε, Eukaryotic; Vol.1 - Pages 716-719, Yasuo Kawasaki and Akio Sugino

DNA Polymerase I, Bacterial; Vol.1 - Pages 720-725, Catherine M. Joyce

DNA Polymerase II, Bacterial; Vol.1 - Pages 726-728, Judith L. Campbell

DNA Polymerase III, Bacterial; Vol.1 - Pages 729-733, Hisaji Maki

DNA Polymerases: Kinetics and Mechanism; Vol.1 - Pages 734-739, Kenneth A. Johnson

DNA Replication Fork, Bacterial; Vol.1 - Pages 740-744, Nancy G. Nossal

DNA Replication Fork, Eukaryotic; Vol.1 - Pages 745-748, Lori M. Kelman, Jerard Hurwitz and Zvi Kelman

DNA Replication, Mitochondrial; Vol.1 - Pages 749-752, David A. Clayton

DNA Replication: Eukaryotic Origins and the Origin Recognition Complex; Vol.1 - Pages 753-760, Melvin L. DePamphilis and Cong-jun Li

DNA Replication: Initiation in Bacteria; Vol.1 - Pages 761-766, Jon M. Kaguni

DNA Restriction and Modification: Type I Enzymes; Vol.1 - Pages 767-771, David T. F. Dryden

DNA Restriction and Modification: Type II Enzymes; Vol.1 - Pages 772-777, Darren M. Gowers and Stephen E. Halford

DNA Restriction and Modification: Type III Enzymes; Vol.1 - Pages 778-781, Desirazu N. Rao and S. Srivani

DNA Secondary Structure; Vol.1 - Pages 782-787, Albino Bacolla and Robert D. Wells

DNA Sequence Recognition by Proteins; Vol.1 - Pages 788-793, Arabela A. Grigorescu and John M. Rosenberg

DNA Supercoiling; Vol.1 - Pages 794-797, Tao-shih Hsieh

DNA Topoisomerases: Type I; Vol.1 - Pages 798-805, James J. Champoux

DNA Topoisomerases: Type II; Vol.1 - Pages 806-811, Renier Velez-Cruz and Neil Osheroff

DNA Topoisomerases: Type III–RecQ Helicase Systems; Vol.1 - Pages 812-816, Rodney Rothstein and Erika Shor

EF-G and EF-Tu Structures and Translation Elongation in Bacteria; Vol.2 - Pages 1-5, Poul Nissen and Jens Nyborg

Exonucleases, Bacterial; Vol.2 - Pages 66-72, Susan T. Lovett

HIV-1 Reverse Transcriptase Structure; Vol.2 - Pages 388-392, Kalyan Das, Stefan G. Sarafianos, Eddy Arnold and Stephen H. Hughes

Homologous Recombination in Meiosis; Vol.2 - Pages 393-397, Nancy M. Hollingsworth

lac Operon; Vol.2 - Pages 529-534, Liskin Swint-Kruse and Kathleen S. Matthews

LexA Regulatory System; Vol.2 - Pages 546-550, Veronica G. Godoy, Penny J. Beuning and Graham C. Walker

Metaphase Chromosome; Vol.2 - Pages 666-671, Sharron Vass and Margarete M. S. Heck

Methyl-CpG-Binding Proteins; Vol.2 - Pages 672-675, David G. Skalnik

mRNA Polyadenylation in Eukaryotes; Vol.2 - Pages 748-752, Mary Edmonds

mRNA Processing and Degradation in Bacteria; Vol.2 - Pages 753-757, Deborah A. Steege

Non-Homologous End Joining; Vol.3 - Pages 66-70, Penny A. Jeggo

Nonhomologous Recombination: Bacterial Transposons; Vol.3 - Pages 71-79, Bao Ton Hoang and Michael G. Chandler

Nonhomologous Recombination: Retrotransposons; Vol.3 - Pages 80-86, Siew Loon Ooi and Jef D. Boeke

Nuclear Organization, Chromatin Structure, and Gene Silencing; Vol.3 - Pages 105-108, Lori L. Wallrath, John R. Danzer, Oya Yazgan and Pamela K. Geyer

Nucleoid Organization of Bacterial Chromosomes; Vol.3 - Pages 115-118, Charles J. Dorman

Nucleolus, Overview; Vol.3 - Pages 119-122, Thoru Pederson

Nucleotide Excision Repair and Human Disease; Vol.3 - Pages 123-129, James E. Cleaver

Nucleotide Excision Repair in Eukaryotes; Vol.3 - Pages 130-133, Laura A. Lindsey-Boltz and Aziz Sancar

Nucleotide Excision Repair, Bacterial: The UvrABCD System; Vol.3 - Pages 134-142, Bennett Van Houten and Lawrence Grossman

Nucleotide Excision Repair: Biology; Vol.3 - Pages 143-147, Errol C. Friedberg

PHO Regulon; Vol.3 - Pages 262-265, Bengt L. Persson

Pre-tRNA and Pre-rRNA Processing in Bacteria; Vol.3 - Pages 420-424, Sidney R. Kushner

Pre-tRNA and Pre-rRNA Processing in Eukaryotes; Vol.3 - Pages 425-431, Hendrik A. Raue

Prions and Epigenetic Inheritance; Vol.3 - Pages 432-436, Reed B. Wickner

Processivity Clamps in DNA Replication: Clamp Loading; Vol.3 - Pages 441-446, Megan J. Davey and Mike O'Donnell

Recombination: Heteroduplexand Mismatch Repair in vitro; Vol.3 - Pages 645-648, Leroy Worth, Jr.

Recombination: Strand Transferases; Vol.3 - Pages 649-653, Floyd R. Bryant

Recombination-Dependent DNA Replication; Vol.3 - Pages 654-659, Kenneth N. Kreuzer

recQ DNA Helicase Family in Genetic Stability; Vol.3 - Pages 660-664, Mary A. Risinger and Joanna Groden

Reverse Transcriptase and Retroviral Replication; Vol.3 - Pages 708-713, Laura Tarrago-Litvak, Marie-Line Andreola and Simon Litvak

Ribosome Assembly; Vol.3 - Pages 719-724, John L. Woolford

Ribosome Structure; Vol.3 - Pages 725-732, Brian T. Wimberly

Ribozyme Mechanisms; Vol.3 - Pages 733-737, John Hsieh and Carol A. Fierke

Ribozyme Structural Elements: Group I Introns; Vol.3 - Pages 738-742, Barbara L. Golden

Ribozyme Structural Elements: Hairpin Ribozyme; Vol.3 - Pages 743-746, Adrian R. Ferre-D'Amare

Ribozymes and Evolution; Vol.3 - Pages 747-752, Niles Lehman

RNA Editing; Vol.3 - Pages 753-758, Charles E. Samuel

RNA Polymerase I and RNA Polymerase III in Eukaryotes; Vol.3 - Pages 759-762, Robert J. White

RNA Polymerase II and Basal Transcription Factors in Eukaryotes; Vol.3 - Pages 763-765, Joan Weliky Conaway and Ronald C. Conaway

RNA Polymerase II Elongation Control in Eukaryotes; Vol.3 - Pages 766-769, David H. Price

RNA Polymerase II Structure in Eukaryotes; Vol.3 - Pages 770-774, Patrick Cramer

RNA Polymerase Reaction in Bacteria; Vol.3 - Pages 775-780, Arkady A. Mustaev and Alexander D. Goldfarb

RNA Polymerase Structure, Bacterial; Vol.3 - Pages 781-784, Michael Anikin, Dmitri Temiakov and William T. McAllister

Sigma Factors; Vol.4 - Pages 41-44, John D. Helmann

Sliding Clamps in DNA Replication: E. coli -Clamp and PCNA Structure; Vol.4 - Pages 45-47, Eric R. Goedken and John Kuriyan

Spliceosome; Vol.4 - Pages 88-92, Timothy W. Nilsen

T7 RNA Polymerase; Vol.4 - Pages 147-151, Rui Sousa

Telomeres: Maintenance and Replication; Vol.4 - Pages 174-179, Alessandro Bianchi and David Shore

Transcription Termination; Vol.4 - Pages 195-199, Thomas J. Santangelo and Jeffrey W. Roberts

Transcription-Coupled DNA Repair, Overview; Vol.4 - Pages 204-208, Isabel Mellon

Translation Elongation in Bacteria; Vol.4 - Pages 214-223, Oliver Vesper and Knud H. Nierhaus

Translation Elongation in Eukaryotes; Vol.4 - Pages 224-229, William C. Merrick and Anton A. Komar

Translation Initiation in Bacteria: Factors and Mechanisms; Vol.4 - Pages 230-236, Cynthia L. Pon and Claudio O. Gualerzi

Translation Initiation in Eukaryotes: Factors and Mechanisms; Vol.4 - Pages 237-241, Tatyana V. Pestova and Christopher U. T. Hellen

Translation Termination and Ribosome Recycling; Vol.4 - Pages 242-246, Nadja Koloteva-Levin and Mick F. Tuite

Translesion DNA Polymerases, Eukaryotic; Vol.4 - Pages 247-250, Alexandra Vaisman and Roger Woodgate

tRNA Synthetases; Vol.4 - Pages 263-266, Karla L. Ewalt and Paul Schimmel

trp Operon and Attenuation; Vol.4 - Pages 267-271, Paul Gollnick

UmuC, D Lesion Bypass DNA Polymerase V; Vol.4 - Pages 308-312, Zvi Livneh

XPV DNA Polymerase and Ultraviolet Damage Bypass; Vol.4 - Pages 419-421, Alan R. Lehmann

Yeast GAL1–GAL10 System; Vol.4 - Pages 429-433, Dennis Lohr and Ralph Bash

**Signaling**

Abscisic Acid (ABA); Vol.1 - Pages 6-11, Ramanjulu Sunkar and Jian-Kang Zhu

Adenosine Receptors; Vol.1 - Pages 34-39, Lauren J. Murphree and Joel Linden

Adenylyl Cyclases; Vol.1 - Pages 40-45, Ronald Taussig

Adrenergic Receptors; Vol.1 - Pages 46-50, David B. Bylund

A-Kinase Anchoring Proteins; Vol.1 - Pages 64-67, Lorene K. Langeberg and John D. Scott

Angiotensin Receptors; Vol.1 - Pages 111-115, Tadashi Inagami

ARF Family; Vol.1 - Pages 120-122, Gustavo Pacheco-Rodriguez, Joel Moss and Martha Vaughan

B-Cell Antigen Receptor; Vol.1 - Pages 155-158, Thomas M. Yankee and Edward A. Clark

Bradykinin Receptors; Vol.1 - Pages 182-185, Ronald M. Burch

Brassinosteroids; Vol.1 - Pages 192-197, Steven D. Clouse

Cadherin Signaling; Vol.1 - Pages 199-204, David B. Sacks and Jonathan M. G. Higgins

Calcitonin Gene-Related Peptide and Adrenomedullin Receptors; Vol.1 - Pages 212-216, Debbie L. Hay, Alex C. Conner and David R. Poyner

Calcitonin Receptor; Vol.1 - Pages 217-220, Samia I. Girgis, Niloufar Moradi-Bidhendi, Lucia Mancini and Iain MacIntyre

Calcium/Calmodulin-Dependent Protein Kinases; Vol.1 - Pages 281-286, J. Robison and Roger J. Colbran

c-fes Proto-Oncogene; Vol.1 - Pages 377-382, Thomas E. Smithgall and Robert I. Glazer

Chemokine Receptors; Vol.1 - Pages 413-418, Ann Richmond and Guo-Huang Fan

Chemotactic Peptide/Complement Receptors; Vol.1 - Pages 425-429, Eric R. Prossnitz and Larry A. Sklar

Cyclic AMP Receptors of Dictyostelium; Vol.1 - Pages 488-493, Dale Hereld and Peter N. Devreotes

Cyclic GMP Phosphodiesterases; Vol.1 - Pages 494-500, Sharron H. Francis and Jackie D. Corbin

Cyclic Nucleotide Phosphodiesterases; Vol.1 - Pages 501-505, Vincent C. Manganiello and Eva Degerman

Cyclic Nucleotide-Dependent Protein Kinases; Vol.1 - Pages 506-511, Sharron H. Francis and Jackie D. Corbin

Cyclic Nucleotide-Regulated Cation Channels; Vol.1 - Pages 512-515, Martin Biel and Franz Hofmann

Cytokines; Vol.1 - Pages 550-555, Andrea L. Wurster and Michael J. Grusby

Cytokinin; Vol.1 - Pages 562-567, Thomas Schmulling

Diacylglycerol Kinases and Phosphatidic Acid Phosphatases; Vol.1 - Pages 593-597, Stephen M. Prescott and Matthew K. Topham

Dopamine Receptors; Vol.1 - Pages 817-822, Kim A. Neve

Eicosanoid Receptors; Vol.2 - Pages 6-9, Richard M. Breyer and Matthew D. Breyer

Endocannabinoids; Vol.2 - Pages 13-15, Daniele Piomelli

Epidermal Growth Factor Receptor Family; Vol.2 - Pages 51-55, Denis Tvorogov and Graham Carpenter

FAK Family; Vol.2 - Pages 80-84, Steven K. Hanks

Fatty Acid Receptors; Vol.2 - Pages 95-98, Christer Owman and Bjorn Olde

Fibroblast Growth Factor Receptors and Cancer-Associated Perturbations; Vol.2 - Pages 112-117, Marko Kornmann and Murray Korc

G Protein Signaling Regulators; Vol.2 - Pages 147-151, John H. Exton

G Protein-Coupled Receptor Kinases and Arrestins; Vol.2 - Pages 152-157, Jeffrey L. Benovic

G12/G13 Family; Vol.2 - Pages 158-161, Stefan Offermanns

GABAA Receptor; Vol.2 - Pages 162-166, Richard W. Olsen and Gregory W. Sawyer

GABAB Receptor; Vol.2 - Pages 167-170, S. J. Enna

Gi Family of Heterotrimeric G Proteins; Vol.2 - Pages 181-185, Maurine E. Linder

Glucagon Family of Peptides and their Receptors; Vol.2 - Pages 193-196, Laurie L. Baggio and Daniel J. Drucker

Glutamate Receptors, Ionotropic; Vol.2 - Pages 213-219, Derek B. Scott and Michael D. Ehlers

Glutamate Receptors, Metabotropic; Vol.2 - Pages 220-223, P. Jeffrey Conn

Glycine Receptors; Vol.2 - Pages 237-243, Bodo Laube and Heinrich Betz

Glycogen Synthase Kinase-3; Vol.2 - Pages 255-260, James R. Woodgett

Gq Family; Vol.2 - Pages 316-320, Wanling Yang and John D. Hildebrandt

Gs Family of Heterotrimeric G Proteins; Vol.2 - Pages 337-341, Susanne M. Mumby

Hematopoietin Receptors; Vol.2 - Pages 348-353, Barbara A. Miller and Joseph Y. Cheung

Hepatocyte Growth Factor/Scatter Factor Receptor; Vol.2 - Pages 367-371, Selma Pennacchietti and Paolo M. Comoglio

Histamine Receptors; Vol.2 - Pages 378-383, Stephen J. Hill and Jillian G. Baker

Immunoglobulin (Fc) Receptors; Vol.2 - Pages 411-416, Mark Hogarth

Inositol Phosphate Kinases and Phosphatases; Vol.2 - Pages 427-429, Stephen B. Shears

Insulin Receptor Family; Vol.2 - Pages 436-440, Paul F. Pilch and Jongsoon Lee

Integrin Signaling; Vol.2 - Pages 441-445, Lawrence E. Goldfinger and Mark H. Ginsberg

Interferon Receptors; Vol.2 - Pages 446-451, Christopher P. Elco and Ganes C. Sen

JAK-STAT Signaling Paradigm; Vol.2 - Pages 491-496, Edward Cha and Christian Schindler

Leptin; Vol.2 - Pages 541-545, Thomas W. Gettys

Lysophospholipid Receptors; Vol.2 - Pages 602-604, Gabor J. Tigyi

Melanocortin System; Vol.2 - Pages 617-620, Roger D. Cone

Mitogen-Activated Protein Kinase Family; Vol.2 - Pages 737-742, Hidemi Teramoto and J. Silvio Gutkind

Muscarinic Acetylcholine Receptors; Vol.2 - Pages 775-777, Neil M. Nathanson

Natriuretic Peptides and their Receptors; Vol.3 - Pages 1-5, Lincoln R. Potter

Neuropeptide Y Receptors; Vol.3 - Pages 26-31, Eric M. Parker

Neurotensin Receptors; Vol.3 - Pages 32-36, William Rostene, Patrick Kitabgi and Didier Pelaprat

Neurotransmitter Transporters; Vol.3 - Pages 37-40, Aurelio Galli, Randy D. Blakely and Louis J. DeFelice

Neurotrophin Receptor Signaling; Vol.3 - Pages 41-45, Jennifer J. Gentry and Bruce D. Carter

Nicotinic Acetylcholine Receptors; Vol.3 - Pages 57-61, Nivalda O. Rodrigues-Pinguet and Henry A. Lester

Nitric Oxide Signaling; Vol.3 - Pages 62-65, Michael A. Marletta

Nuclear Factor kappaB; Vol.3 - Pages 96-99, Thomas D. Gilmore

Olfactory Receptors; Vol.3 - Pages 149-154, Sigrun I. Korsching

Opioid Receptors; Vol.3 - Pages 167-171, P. Y. Law and Horace H. Loh

P2X Purinergic Receptors; Vol.3 - Pages 183-187, Annmarie Surprenant

P2Y Purinergic Receptors; Vol.3 - Pages 188-191, George R. Dubyak

p53 Protein; Vol.3 - Pages 192-195, Jamie Hearnes and Jennifer Pietenpol

p70 S6 Kinase/mTOR; Vol.3 - Pages 196-200, Christopher G. Proud

Parathyroid Hormone/Parathyroid Hormone-Related Protein Receptor; Vol.3 - Pages 201-207, Thomas J. Gardella

Peroxisome Proliferator-Activated Receptors; Vol.3 - Pages 239-245, Mary C. Sugden, Edward A. Sugden and Mark J. Holness

Pheromone Receptors (Yeast); Vol.3 - Pages 256-261, James B. Konopka and Jeremy W. Thorner

Phosphatidylinositol Bisphosphate and Trisphosphate; Vol.3 - Pages 266-271, Alex Toker

Phosphoinositide 3-Kinase; Vol.3 - Pages 281-286, Khatereh Ahmadi and Michael Waterfield

Phosphoinositide 4- and 5-Kinases and Phosphatases; Vol.3 - Pages 287-291, Shawn F. Bairstow, Matthew W. Bunce and Richard A. Anderson

Phosphoinositide-Dependent Protein Kinases; Vol.3 - Pages 292-296, Nick R. Leslie and C. Peter Downes

Phospholipase A2; Vol.3 - Pages 297-300, Timothy R. Smith and Edward A. Dennis

Phospholipase C; Vol.3 - Pages 301-305, Fujio Sekiya, Yeun Ju Kim and Sue Goo Rhee

Phospholipase D; Vol.3 - Pages 306-313, Mary M. LaLonde and Michael A. Frohman

Photoreceptors; Vol.3 - Pages 326-329, King-Wai Yau

Plant Signaling: Peptides; Vol.3 - Pages 381-384, Clarence A. Ryan and Gregory Pearce

Platelet-Activating Factor Receptor; Vol.3 - Pages 394-398, Katherine M. Howard and Merle S. Olson

Platelet-Derived Growth Factor Receptor Family; Vol.3 - Pages 399-406, Marina Kovalenko and Andrius Kazlauskas

Protein Kinase B; Vol.3 - Pages 516-522, Bettina A. Dummler and Brian A. Hemmings

Protein Kinase C Family; Vol.3 - Pages 523-526, Alexandra C. Newton

Protein Tyrosine Phosphatases; Vol.3 - Pages 536-542, David J. Pagliarini, Fred L. Robinson and Jack E. Dixon

Proteinase-Activated Receptors; Vol.3 - Pages 543-548, Kristina K. Hansen and Morley D. Hollenberg

Rab Family; Vol.3 - Pages 629-634, Mary W. McCaffrey and Andrew J. Lindsay

Ran GTPase; Vol.3 - Pages 635-639, Mary Shannon Moore

Ras Family; Vol.3 - Pages 640-644, Lawrence A. Quilliam

Retinoblastoma Protein (pRB); Vol.3 - Pages 700-703, Nicholas Dyson and Maxim Frolov

Retinoic Acid Receptors; Vol.3 - Pages 704-707, Martin Petkovich

Serine/Threonine Phosphatases; Vol.4 - Pages 27-32, Thomas S. Ingebritsen

Serotonin Receptor Signaling; Vol.4 - Pages 33-37, Paul J. Gresch and Elaine Sanders-Bush

Small GTPases; Vol.4 - Pages 48-54, Adam Shutes and Channing J. Der

Somatostatin Receptors; Vol.4 - Pages 55-60, Agnes Schonbrunn

Src Family of Protein Tyrosine Kinases; Vol.4 - Pages 93-98, Jonathan A. Cooper

Steroid/Thyroid Hormone Receptors; Vol.4 - Pages 111-116, Ramesh Narayanan and Nancy L. Weigel

Syk Family of Protein Tyrosine Kinases; Vol.4 - Pages 139-145, Andrew C. Chan

Tachykinin/Substance P Receptors; Vol.4 - Pages 152-157, Mark D. Richardson and Madan M. Kwatra

Taste Receptors; Vol.4 - Pages 158-161, John D. Boughter, Jr. and Steven D. Munger

T-Cell Antigen Receptor; Vol.4 - Pages 162-168, Andrea L. Szymczak and Dario A. A. Vignali

Tec/Btk Family Tyrosine Kinases; Vol.4 - Pages 169-173, Shuling Guo and Owen N. Witte

Thyroid-Stimulating Hormone/Luteinizing Hormone/Follicle-Stimulating Hormone Receptors; Vol.4 - Pages 180-186, Deborah L. Segaloff, Dario Mizrachi and Mario Ascoli

Toll-Like Receptors; Vol.4 - Pages 190-194, Himanshu Kumar, Kiyoshi Takeda and Shizuo Akira

Transforming Growth Factor-β Receptor Superfamily; Vol.4 - Pages 209-213, Mark de Caestecker

Tumor Necrosis Factor Receptors; Vol.4 - Pages 277-283, Karen G. Potter and Carl F. Ware

Vascular Endothelial Growth Factor Receptors; Vol.4 - Pages 337-342, Kenneth A. Thomas

Vasopressin/Oxytocin Receptor Family; Vol.4 - Pages 343-348, Michael J. Brownstein

Vitamin D Receptor; Vol.4 - Pages 378-383, Diane R. Dowd and Paul N. MacDonald

Von Hippel-Lindau (VHL) Protein; Vol.4 - Pages 416-418, Ronald C. Conaway and Joan Weliky Conaway

## Techniques and Methodology

Affinity Chromatography; Vol.1 - Pages 51-56, Pedro Cuatrecasas and Meir Wilchek

Affinity Tags for Protein Purification; Vol.1 - Pages 57-63, Joseph J. Falke and John A. Corbin

Genome-Wide Analysis of Gene Expression; Vol.2 - Pages 175-180, Karine G. Le Roch and Elizabeth A. Winzeler

HPLC Separation of Peptides; Vol.2 - Pages 398-403, James D. Pearson

Imaging Methods; Vol.2 - Pages 405-410, Gyorgy Szabadkai and Rosario Rizzuto

Inorganic Biochemistry; Vol.2 - Pages 417-420, Robert J. P. Williams

Multiple Sequence Alignment and Phylogenetic Trees; Vol.2 - Pages 770-774, Russell F. Doolittle

Oligosaccharide Analysis by Mass Spectrometry; Vol.3 - Pages 155-160, Andrew J. Hanneman and Vernon N. Reinhold

PCR (Polymerase Chain Reaction); Vol.3 - Pages 208-210, Michael J. Brownstein

Polysialic Acid inMolecular Medicine; Vol.3 - Pages 407-414, Frederic A. Troy, II

Protein Data Resources; Vol.3 - Pages 478-483, Philip E. Bourne

Secondary Structure in Protein Analysis; Vol.4 - Pages 1-6, George D. Rose

Spectrophotometric Assays; Vol.4 - Pages 67-75, Britton Chance

Two-Dimensional Gel Electrophoresis; Vol.4 - Pages 284-289, Gerhard Schmid, Denis Hochstrasser and Jean-Charles Sanchez

X-Ray Determination of 3-D Structure in Proteins; Vol.4 - Pages 422-428, Martha L. Ludwig