3

Control of Protein Function

In the cell, precise regulation of protein function is essential to avoid chaos. This chapter describes the most important molecular mechanisms by which protein function is regulated in cells. These range from control of a protein's location and lifetime within the cell to the binding of regulatory molecules and covalent modifications such as phosphorylation that rapidly switch protein activity on or off. Also covered here are the nucleotide-driven switches in conformation that underlie the action of motor proteins and that regulate many signal transduction pathways.

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3-0 Overview: Mechanisms of Regulation

Protein function in living cells is precisely regulated

A typical bacterial cell contains a total of about 250,000 protein molecules (comprising different amounts of each of several thousand different gene products), which are packed into a volume so small that it has been estimated that, on average, they are separated from one another by a distance that would contain only a few molecules of water. It is likely that eukaryotic cells are at least as densely packed. In this crowded environment, precise regulation of protein function is essential to avoid chaos. Regulation of protein function *in vivo* tends to occur by many different mechanisms, which fall into several general classes. Protein function can be controlled by localization of the gene product and/or the species it interacts with, by the covalent or noncovalent binding of **effector** molecules, and by the amount and lifetime of the active protein.

Proteins can be targeted to specific compartments and complexes

Not all proteins are absolutely specific, and many also have more than one function. Consequently, it is often undesirable to have such proteins distributed everywhere in the cell, where they may carry out unwanted reactions. A simple way to regulate their activity is to ensure that the protein is only present in its active form in the specific compartment where it is needed, or when bound in a complex with other macromolecules that participate in its function. There are many ways in which specific localization can be achieved. Proteins can be targeted to cellular compartments by so-called signal sequences that are an intrinsic part of the encoded amino-acid sequence, or by attachment of, for example, a lipid tail that inserts into membranes. They can be directed to a complex of interacting proteins by a structural *interaction domain* that recognizes some covalent modification such as phosphorylation on another protein. Localization is a dynamic process and a given protein may be targeted to different compartments at different stages of the cell cycle: many transcription factors, for example, cycle between the nucleus and the cytosol in response to extracellular signals. When the protein is not in the location where it is needed, very often it is maintained in an inactive conformation.

Protein activity can be regulated by binding of an effector and by covalent modification

Protein activity can also be controlled by the binding of effector molecules, which often work by inducing conformational changes that produce inactive or active forms of the protein. Effectors may be as small as a proton or as large as another macromolecule. Effectors may bind noncovalently or may modify the covalent structure of the protein, reversibly or irreversibly. Effectors that regulate activity by binding to the active site usually take the form of inhibitors that compete with the substrate for binding. Often, the product of an enzyme reaction can act as such a **competitive inhibitor**, allowing the enzyme to regulate itself when too much product might be made. Ligands, including reaction products, may also bind to sites remote from the active site and in so doing either activate or inhibit a protein. Proteins regulated in this way tend to be oligomeric and *allosteric*. Allosteric proteins have multiple ligand-binding sites and these show *cooperativity* of binding: in *positive cooperativity* the first ligand molecule to bind is bound weakly, but its binding alters the conformation of the protein in such a way that binding of the second and subsequent ligand molecules is promoted. Cooperativity may also be negative: the first ligand binding weakens and thereby effectively inhibits subsequent binding to the other sites. Metabolic pathways often employ allosteric effectors as part of a feedback control mechanism: the end product of the pathway acts as an allosteric inhibitor of one of the earlier

Definitions

competitive inhibitor: a species that competes with substrate for binding to the active site of an enzyme and thus inhibits catalytic activity.

effector: a species that binds to a protein and modifies its activity. Effectors may be as small as a proton or as large as a membrane and may act by covalent binding, noncovalent binding, or covalent modification.

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enzymes in the pathway, so when too much of this product is synthesized it feeds back and shuts off one of the enzymes that help make it, as we shall see later in this chapter.

Binding of effector molecules can be covalent or can lead to covalent changes in a protein. The most common form of post-translational covalent modification is reversible phosphorylation on the hydroxyl group of the side chains of serine, threonine or tyrosine residues, but many other modifications are known, including side-chain methylation, covalent attachment of carbohydrates and lipids, amino-terminal acetylation and limited proteolytic cleavage, in which proteases cut the polypeptide chain in one or more places. Modifications such as phosphorylation or proteolytic cleavage may either activate or inactivate the protein.

Signal amplification is an essential feature in the control of cell function and covalent modification of proteins is the way such amplification is usually achieved. Often, an extracellular stimulus is of short duration and involves only a very low concentration, or a small change in concentration, of a hormone or regulatory molecule. Yet the cellular response must not only be very rapid; in many cases it must be massive, including a change in activities of many enzymes and alteration in the transcription of many genes. Covalent modification of a protein provides a simple mechanism by which a regulatory signal can produce a very large output. A single molecule of an enzyme whose catalytic activity is turned on by a covalent modification can process many thousands of substrate molecules. And if that substrate is another enzyme, the amplification is further magnified. When covalent modification of one enzyme causes it to become active so that it can, in turn, covalently modify and activate another enzyme and so on, a regulatory cascade is set up that leads to enormous, rapid changes in the final output. Blood clotting is an example of such an amplification cascade based on proteolysis.

Protein activity may be regulated by protein quantity and lifetime

The activity of a protein can also be regulated by controlling its amount and lifetime in the cell. This control may be exercised at several places in the flow of information from gene to protein. At its simplest, the amount of protein can be set by the level of transcription, which in turn can be controlled by, for example, the strength of the promoter or the action of a transcription factor, which may be a repressor or activator. The level of mRNA may also be adjusted after transcription by varying the rate of RNA degradation. At the level of the protein, quantities are controlled by the lifetime of the molecule, which is determined by its rate of degradation. The rate of turnover varies considerably from protein to protein; there are several specific mechanisms for targeting protein molecules to degradative machinery in the cell, including covalent attachment of the small protein ubiquitin.

A single protein may be subject to many regulatory influences

These various strategies are not mutually exclusive and any one protein may be subject to several of them. Coordination and integration of regulatory signals is achieved largely through signal transduction networks that set the balance of activities and thereby the balance of metabolism and cell growth and division pathways. Integration of signaling pathways is achieved through proteins, such as protein kinases, whose activity is under the control of several different mechanisms that can be independently regulated by incoming signals, as in the example of the protein tyrosine kinases known as cyclin-dependent kinases (CDKs), which control a eukaryotic cell's progression through the cell cycle (Figure 3-1).

Wyrick, J.J. and Young, R.A.: **Deciphering gene expression regulatory networks.** *Curr. Opin. Genet. Dev.* 2002, **12**:130–136.

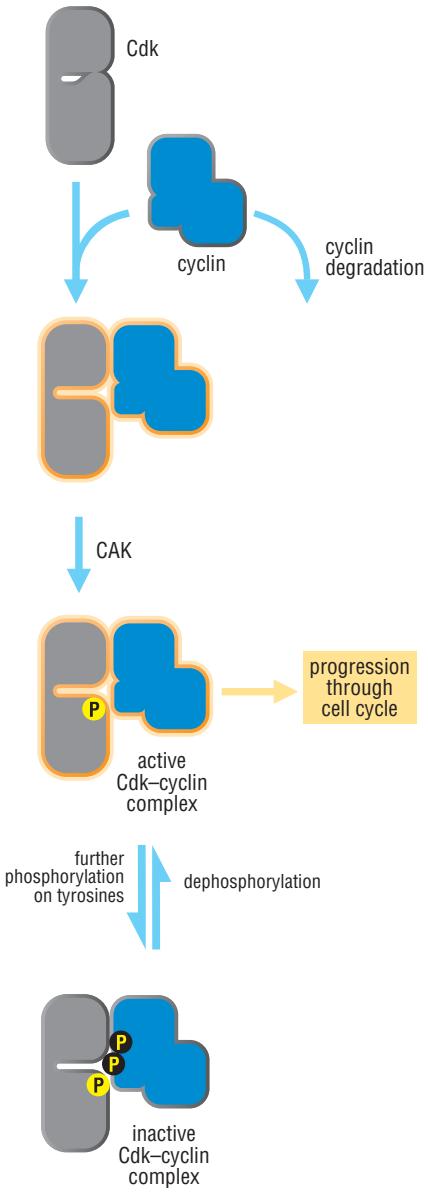

Figure 3-1 The cyclin-dependent protein kinases that control progression through the cell cycle are regulated by a number of different mechanisms Activation of a cyclin-dependent kinase (Cdk) at the appropriate point in the cell cycle requires both binding by its cyclin ligand, which induces a conformational change in the Cdk, phosphorylation of the Cdk in this Cdk–cyclin complex at a particular tyrosine residue, and dephosphorylation at one or two other tyrosines (depending on the particular Cdk). Cyclin binding is determined by the levels of cyclin present in the cell, which are strictly controlled in a temporal fashion by a signaling network that regulates gene transcription and protein degradation. Phosphorylation at the activating site is carried out by a so-called Cdk-activating kinase (CAK) which is present throughout the cell cycle but cannot act until the Cdk has bound cyclin; phosphorylation and dephosphorylation of the inhibitory tyrosines are carried out by another kinase and a phosphatase, respectively, whose activities are also subject to finely tuned regulation.

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The flow of information within the cell is regulated and integrated by the combinatorial use of small protein domains that recognize specific ligands

Many proteins that regulate cell behavior are constructed in a modular fashion from a number of different small domains with distinct binding specificities and functions (see Figure 1-46). Signal transduction proteins, regulatory proteins of the cell cycle, proteins that carry out targeted proteolysis, and proteins that regulate secretory pathways, cytoskeletal organization or gene expression all come into this category. Many gene regulatory proteins, for example, bind both to DNA and to another protein to carry out their function; they are composed of a domain that binds a specific DNA sequence and a protein-binding domain, which may target another molecule of the same protein (see Figure 1-41) or another gene regulatory protein. In some enzymes, a catalytic domain is attached to one or a number of protein-binding domains. These **interaction domains** (also called recognition modules) target the attached catalytic domain to a particular multiprotein complex or an appropriate subcellular location (such as the nucleus or the plasma membrane). Some interaction domains, such as the calcium-binding EF hand, often act as regulatory domains for enzymes. In signal transduction pathways, active multiprotein complexes are assembled by interaction domains that target the components to the complex. For example, the WD40 domain of the β-subunit of the heterotrimeric G protein targets this subunit to the α and γ subunits (see Figure 3-15). In some cases the interaction domain of a protein may interact with that protein's own catalytic domain in an autoinhibitory manner. Binding of the interaction domain to another protein then relieves the inhibition and activates the enzyme. Some protein kinases are autoregulated this way (see section 3-13).

Interaction domains are independently folded modules, 35–150 residues in length, which can still bind their target ligands if expressed independently of their "host" protein. Their amino and carboxyl termini are close together in space, with the ligand-binding site being located on the opposite face of the module. This allows an interaction domain to be inserted into a loop region of a catalytic domain, for example, as in the case of mammalian phospholipase Cγ, where two SH2 and one SH3 domains are inserted within the catalytic domain without disturbing its fold or having their own binding sites blocked. This type of domain organization also allows different domains to be strung together in combinatorial fashion and still retain their function.

Interaction domains can be divided into distinct families whose members are related by sequence, structure, and ligand-binding properties (Figure 3-2). Different members of a family recognize somewhat different sequences or structures, providing specificity for the proteins into which they are inserted. Examples of interaction domain families are SH3, WW and EVH1, which recognize proline-rich sequences; SH2 and PTB, which recognize phosphotyrosine-containing sequences; and 14-3-3, FHA, PBD and WD40, which bind to phosphoserine and phosphothreonine motifs. These domains are all common in proteins of signal transduction pathways. The PH and FYVE domains that recognize phospholipids are found in several different pathways, including signal transduction pathways and in proteins that control the traffic between internal membrane-bound compartments of the cell. A given protein may contain one or more copies of several different recognition modules, occurring in a different order in different proteins. Among the GTPase-activating proteins, for example, GAP1 has a PH domain after its GAP domain, while p120 GAP has the opposite order. Some interaction domains also form homo- and hetero-oligomers (typically dimers), which creates yet another level of regulatory versatility and specificity.

Figure 3-2 Interaction domains The name of the particular example shown for each family is given below each structure, along with the function and specificity of the domain.

Definitions

interaction domain: a protein domain that recognizes another protein, usually via a specific recognition motif.

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For a catalog of known intracellular protein interaction domains, see http://www.cellsignal.com/reference

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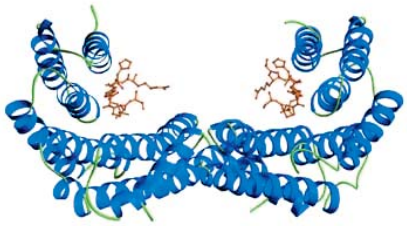
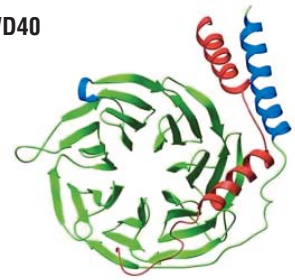
14-3-3



Example: 14-3-3
Function: protein–protein interactions
Specificity: phosphotyrosine

WD40



Example: G protein beta subunit
Function: protein–protein interactions; a stable propeller-like platform to which proteins bind either stably or reversibly
Specificity: various

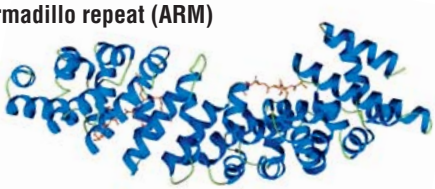
EF-hand

Example: Calmodulin
Function: calcium binding
Specificity: Ca^{2+}

LRR

Example: Rpn1
Function: protein–protein interactions
Specificity: various

Armadillo repeat (ARM)



Example: Importin alpha
Function: protein–protein interactions
Specificity: various

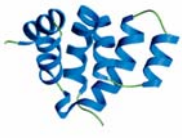
SNARE

Example: SNAP-25B
Function: protein–protein interactions in intracellular membrane fusion
Specificity: other SNARE domains

PTB

Example: Shc
Function: protein–protein interactions
Specificity: phosphotyrosine

Death domain (DD)

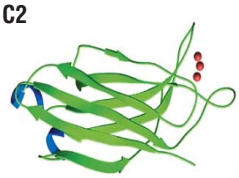


Example: FADD
Function: protein–protein interactions in pathway that triggers apoptosis
Specificity: other DD domains through heterodimers

ANK (ankyrin repeat)

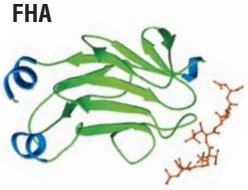
Example: Swi6
Function: protein–protein interactions
Specificity: various

C2



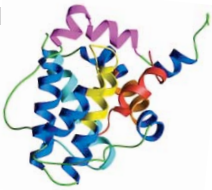
Example: PKC
Function: electrostatic switch
Specificity: phospholipids

FHA



Example: Rad53
Function: protein–protein interactions
Specificity: phosphotyrosine

BH

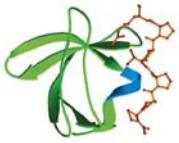


Example: Bcl-Xl
Function: protein–protein interactions
Specificity: Other BH domains through heterodimers

SH2

Example: Src
Function: protein–protein interactions
Specificity: phosphotyrosine

SH3



Example: Sem5
Function: protein–protein interactions
Specificity: proline-rich sequences

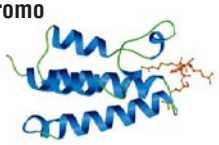
PH

Example: PLC-δ
Function: recruitment of proteins to the membrane
Specificity: phosphoinositides

SAM

Example: EphA4
Function: protein–protein interactions via homo- and heterodimers
Specificity: other SAM domains

Bromo



Example: P/CAF
Function: protein–protein interactions in chromatin remodeling
Specificity: acetylated lysine

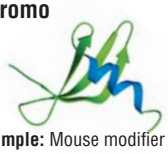
PDZ

Example: PSD-95
Function: protein–protein interactions, often involving transmembrane proteins or ion channels
Specificity: -XXXV/I-COOH

GYF

Example: CD2
Function: protein–protein interactions
Specificity: proline-rich sequences

Chromo



Example: Mouse modifier protein 1
Function: protein–protein interactions in chromatin remodeling
Specificity: methylated lysine

FYVE

Example: Vps27p
Function: Regulation of signaling
Specificity: phosphatidyl-inositol-3-phosphate

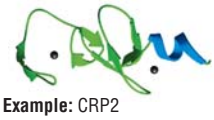
RING finger

Example: c-Cbl
Function: protein–protein interactions in ubiquitin-dependent degradation and transcription regulation
Specificity: various

WW

Example: Pin1
Function: protein–protein interactions
Specificity: proline-rich sequences

LIM



Example: CRP2
Function: protein–protein interactions, usually in transcription regulation
Specificity: various

F-box

Example: Skp2
Function: protein–protein interactions in ubiquitin-dependent protein degradation
Specificity: various

C1

Example: PKC
Function: recruitment of proteins to the membrane
Specificity: phospholipids

Fibronectin

Example: Fibronectin III
Function: protein–protein interactions in cell adhesion to surfaces
Specificity: RGD motif of integrins

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Protein function in the cell is context-dependent

Many cellular processes involve the interaction of two or more macromolecules: signal transduction pathways are a good example. But when one estimates the number of gene products apparently involved in such pathways, it frequently appears that there are too few different proteins to account for all the different specific interactions that must be made. Put another way, the genomes of higher organisms seem to contain too few genes to fulfill all the cellular functions required. The logical conclusion is that many proteins participate in more than one cellular process. But if chaos is not to result from all these activities occurring simultaneously, both temporal and spatial control over a protein's activity must be exercised. Temporal control can be achieved partly by regulating gene expression and protein lifetime. However, it is increasingly clear that spatial context, the precise location within the cell at which a gene product exercises its biochemical function, is a major mechanism for regulating function.

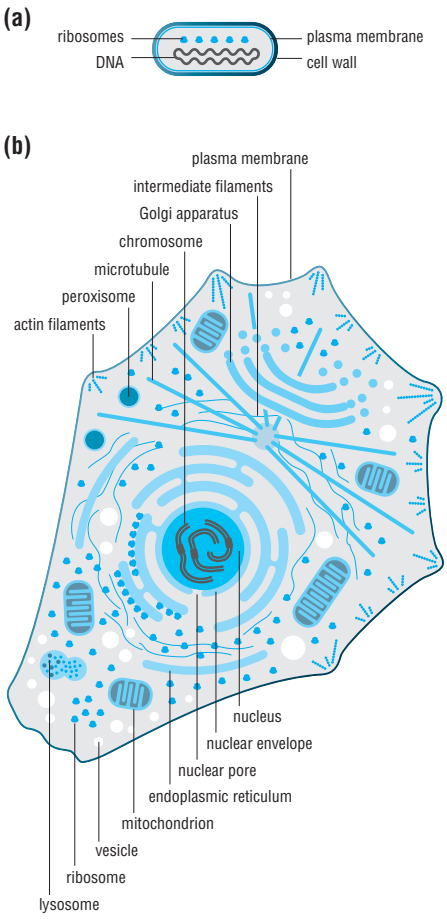

Figure 3-3 The internal structure of cells Schematic diagrams of **(a)** a bacterial cell and **(b)** a typical eukaryotic cell, showing the arrangements of some of the organelles and other internal structures.

It is probable that there is no such thing as a free-floating protein in a eukaryotic cell. Every protein is constrained, whether in a complex with other macromolecules, within a specific organelle, in a cargo vesicle, by attachment to a membrane, or as a passenger on the actin railroads in the cytoskeleton, among others. Moreover, the membrane-bounded vesicles of the cell provide a distinctive environment that can affect protein function, as we discuss in the next section. Prokaryotic cells, which lack organelles and cytoskeletal structures, may be less highly structured, but eukaryotic cells are organized into many compartments (Figure 3-3). In fact, it is increased organization, not increased gene number, that is the real hallmark of eukaryotic cells. For example, the eukaryote fission yeast *Schizosaccharomyces pombe* has fewer genes than the bacterium *Pseudomonas aeruginosa*.

Precise localization of proteins is a central feature of both spatial and temporal organization. In eukaryotic cells, the targeting of proteins to different locations in the cell, such as cytosol versus nucleus, can be regulated according to need, and changes in targeting can modify protein function at the cellular level even when the biochemical function of the protein does not change.

Nowhere is this more evident than in signal transduction pathways. Many of these depend on **protein kinases** which, as discussed later in this chapter, modify protein function by phosphorylating selected amino acids. The number of protein kinase genes in the human genome is large, but is far smaller than the number of potential protein kinase substrates. As we do not appear to possess one kinase for each substrate, kinases must have less than absolute specificity. How then are they prevented from phosphorylating the "wrong" protein at an inappropriate time and place? One answer is to target the kinase to the same location as its "correct" substrate, a location different from that for any other potential substrate; the action of that kinase is then quite specific; and specificity can be altered if required by relocation of kinase and/or substrate.

Another example of control by location is the small monomeric GTPase Tem1 from the budding yeast *Saccharomyces cerevisiae*. Tem1 plays an essential part in terminating the mitotic phase of the cell cycle. The interaction of individual yeast proteins with all the other proteins encoded in the yeast genome has been systematically investigated by two-hybrid analysis (see section 4-4). In such assays Tem1 has been found to interact physically with 24 different yeast gene products. Given its size, no more than about four other proteins could possibly bind to Tem1 at the same time, so what controls which proteins it interacts with at any given time? Differences in the timing of expression of the different target proteins could play some part in determining Tem1's specificity, but binding to specific partners is likely to be mainly controlled by targeting Tem1, and its potential partners, to different locations in the cell at different times.

Definitions

lipid anchor: lipid attached to a protein that inserts into a membrane thereby anchoring the protein to the bilayer.

protein kinase: enzyme that transfers a phosphate group from ATP to the OH group of serines, threonines and tyrosines of target proteins.

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There are several ways of targeting proteins in cells

Targeting of a protein within the cell is achieved in three main ways: by sequences in the protein itself; by various types of post-translational modification; and by binding to a scaffold protein. Localization to the membrane-bounded compartments of the cell—the nucleus, the endoplasmic reticulum (ER), the Golgi complex, and so on—is usually achieved by specific "localization signals" encoded in the protein sequence. For example, the sequence KDEL targets proteins for retention in the endoplasmic reticulum (by binding to a specific receptor) (Figure 3-4a); lysine/arginine-rich clusters such as KRKR target proteins to the nucleus; and the hydrophobic signal sequence found at the beginning of some proteins directs their secretion from the cell. Depending on the organelle to be targeted, localization sequences may be located at either end of the polypeptide chain or even internally. Intrinsic plasma membrane proteins are also targeted to the membrane via the ER by this type of mechanism.

Localization by sequence motifs is an intrinsic property of the protein. In contrast, post-translational targeting mechanisms are regulatable. The commonest mechanism is covalent chemical modification of the protein. Perhaps the most widely used modification of this type is phosphorylation of tyrosine, serine or threonine residues by protein kinases; such modifications target proteins for binding by other proteins that have a recognition site for the modification (Figure 3-4b). This mechanism is particularly important in the formation of signaling complexes and in biochemical switches, and we discuss it in more detail later in this chapter.

Another important post-translational modification targets proteins to the plasma membrane by covalent attachment of their carboxyl or amino terminus to a lipid molecule, often called a **lipid anchor**, which is then inserted into the phospholipid bilayer (Figure 3-4c). For example, the signaling GTPase Ras, which is found mutated in many human tumors, becomes covalently attached to the isoprenoid farnesyl at its carboxyl terminus via a thioester linkage, and this modification, which targets Ras to the cell membrane and thus brings it close to the signaling complexes attached to receptor proteins, is essential for its function in signal transduction (Figure 3-4c).

Although lipid anchors could insert randomly into membranes solely by virtue of their hydrophobicity, evidence is mounting that the membranes in eukaryotic cells are not just random soups of lipids. Membranes appear to have many patches (sometimes called lipid rafts) where specific lipids congregate. These "islands" target particular lipid anchors or lipid-binding domains on proteins not just to the membrane but to very specific places on the membrane. Control of the location and size of these rafts by enzymatic modification and hydrolysis of phospholipids is yet another level of functional organization. We discuss lipid rafts in more detail later, in the section on lipid modification of proteins (see section 3-19), in which we also describe the various types of lipid anchor.

The third common means of protein targeting is by binding to scaffold proteins. These are proteins that can bind several other proteins simultaneously, thereby promoting their interaction (Figure 3-4d). To carry out this function, scaffold proteins generally contain a number of small domains that recognize specific targets, modifications or sequence motifs. One example is the SH3 domain, which binds to proline-rich regions; another is the SH2 domain, which recognizes sequences containing a phosphorylated tyrosine; yet another is the PH domain, which binds to phospholipids in membranes (see section 3-1).

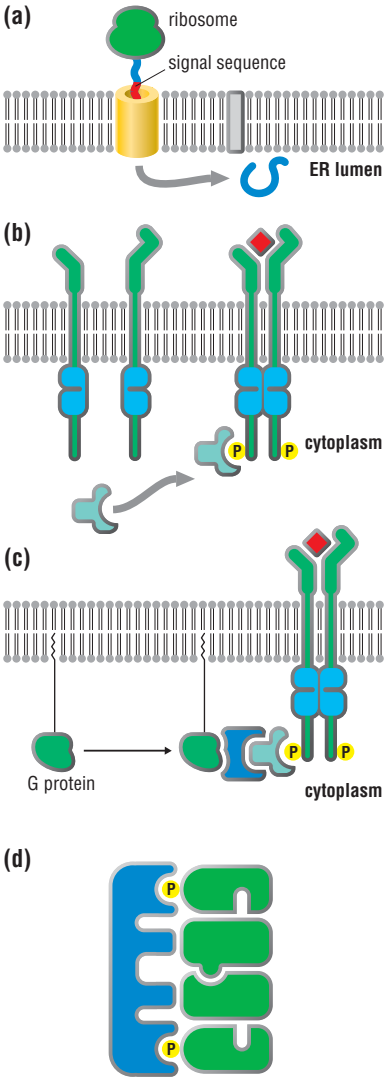

Figure 3-4 Mechanisms for targeting proteins **(a)** Proteins destined for export from the cell are first co-translationally targeted to the endoplasmic reticulum (ER) by signal sequences (red) at the amino terminus of the protein. The signal sequences are cleaved off when protein synthesis is complete, releasing the protein into the lumen of the ER, from which it is exported to the cell surface in transport vesicles. Transmembrane proteins are targeted in a similar way, but have additional internal hydrophobic signal sequences that retain the protein in the membrane. **(b)** Proteins may be targeted to specific signaling complexes by recognizing phosphorylated sites on their target protein. As shown here, a cell-surface receptor (blue and green) with intrinsic protein kinase activity dimerizes on binding an external ligand (red), and this triggers phosphorylation of the cytoplasmic domains. That generates a target site for a cytoplasmic signaling molecule (light blue) that recognizes the phosphorylated residue and binds to the receptor tail. **(c)** A lipid tail is attached to a small G protein (green), thereby anchoring it in the plasma membrane where it can interact with other membrane-bound signaling proteins. It is shown here interacting with an adaptor protein (dark blue) that is part of the signaling complex that builds up around phosphorylated receptor domains. **(d)** A scaffold protein (blue) binds to several different signaling molecules (green) and thereby targets their activities: the signaling molecules may, for example, be protein kinases that can now sequentially phosphorylate one another as part of a signaling pathway.

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Protein function is modulated by the environment in which the protein operates

All proteins are adapted to fold and function optimally in the particular environment of the cellular compartment in which they operate. The cellular aqueous solution is highly viscous and contains many components besides proteins at high concentration, including ions, free polar organic molecules and, most important, the dissociated conjugate acid/conjugate base components of water: the proton and the hydroxide ion. If these two components are present at approximately equal concentration, as is the case in the cytosol of most cells, the solution is neutral with a pH of about 7. If protons are in excess, the solution is acidic, with a lower pH, and ionizable groups tend to be protonated. If hydroxide ions predominate, the solution is alkaline with a pH > 7 and ionizable groups will tend to be deprotonated. Distinct membrane-bounded compartments inside the cell often have a distinct internal microenvironment and the extracellular environment represents a different aqueous environment again from that of the interior. We describe here some examples of the adaptations of proteins for the environments in which they function.

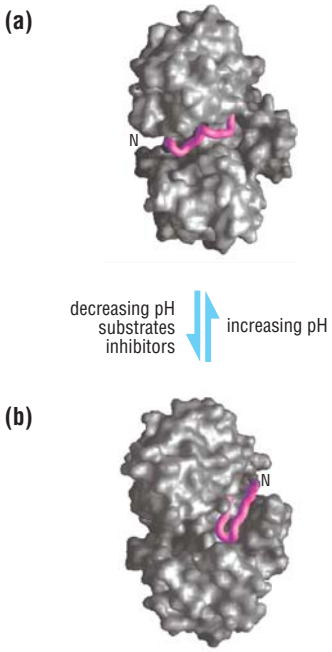

Figure 3-5 Cathepsin D conformational switching Cathepsin D undergoes a pH-dependent conformational change. At the neutral pH of the cytosol **(a)**, the amino-terminal peptide interacts with the active site and blocks substrate access. At the low pH of the endosome **(b)**, in which cathepsin D is active, the amino-terminal segment reorients, opening the active site. At the same time, the low pH sets the protonation states of the catalytic residues for activity. Graphic kindly provided by John Erickson. From Lee, A.Y. *et al.*: *Nat. Struct. Biol.* 1998, **5**:866–871, with permission.

Changes in redox environment can greatly affect protein structure and function

The interiors of cells are for the most part reducing environments: they furnish electrons, often in the form of hydrogen atoms. On the other hand, outside the cell, proteins and small molecules are typically exposed to an oxidizing environment in which electrons can be lost. The chief effect of this difference is that cysteine residues in proteins are usually fully reduced to –SH groups inside the cell, but are readily oxidized to disulfide S–S bridges when a protein is secreted. Cells can exploit this difference to trigger oligomerization by S–S bond formation in secreted proteins, or subunit dissociation and conformational changes when proteins are internalized. For instance, acetylcholinesterase is synthesized as a monomer, but when secreted from muscle and nerve cells self-associates to form dimers and tetramers. These oligomers, which are more stable than the monomer and thus better able to survive outside the cell, are composed of covalently linked subunits. Each monomer has a carboxy-terminal cysteine residue which forms an intersubunit S–S bond with the identical residue on a neighboring polypeptide chain.

Changes in pH can drastically alter protein structure and function

Most cytosolic fluid is maintained at near neutral pH, so that neither acids nor bases predominate. However, there are specialized compartments, such as endosomal vesicles, where the pH is quite acidic. As the surfaces of soluble proteins are chiefly composed of polar side chains, many of which are ionizable, both the net charge on a protein and the distribution of charge over the surface can vary considerably with pH.

If ligand binding depends on electrostatic interactions (see Figure 2-23), changes in the external pH (or ion concentration) can greatly influence binding strength by directly altering the ionization states of groups that interact with the ligand or of groups on the ligand itself. Modulation of the surface charge distribution of a protein by pH changes can also affect the biochemical function indirectly, by changing the extent of ionization of essential functional groups in an active site or binding site through long-range electrostatic interactions. For instance, endosomal proteases, which degrade internalized proteins, are only catalytically active

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at acidic pH, when the charged groups in their active sites are in the proper ionization states. Endosomal proteases are implicated in the degradation of internalized regulatory peptides involved in the control of metabolic pathways and in the processing of intracellular antigens for cytolytic immune responses. Processing occurs in endocytic vesicles whose acidic internal environment is regulated by the presence of an ATP-dependent proton pump. The acidic environment (pH ~ 5) modulates protease activity, protein unfolding and receptor–ligand interactions. The endosomal compartment of liver cells contains an acidic endopeptidase, cathepsin D, that hydrolyzes internalized insulin and generates the primary end-product of degradation. The protease is only active at low pH because its active site contains two essential aspartic acid residues, one of which must be protonated for catalysis to occur.

However, there is an additional aspect to the pH-dependent regulation of cathepsin D activity. The conformation of the protein, particularly the amino terminus, differs at low and high pH. High pH results in a concerted set of conformational changes including the relocation of the amino terminus into the active site, where it blocks substrate binding; also, at this pH both catalytic aspartates are likely to be deprotonated and therefore inactive; their charge state also stabilizes this conformation of the amino terminus. Activation at low pH is due to the protonation of one of the two catalytic aspartates, weakening their electrostatic interactions with the amino-terminal peptide. The released amino-terminal peptide restores the accessibility of the active site to substrate (Figure 3-5).

In some cases, the effect of pH has been exploited to change the structure of a protein completely in an acidic compartment. For example, diphtheria toxin, a protein that is among the deadliest substances known, is synthesized in the neutral pH of the cytosol of the bacterial cell as a single polypeptide chain with three domains: from amino to carboxyl terminus these are designated as domains B, T and A. The B domain binds to a receptor in the target-cell membrane, leading to uptake of the toxin by receptor-mediated endocytosis. The A domain, which is joined to the B domain by a disulfide bond, is an enzyme that kills cells by catalyzing the ADP-ribosylation of elongation factor 2 on the ribosome, leading to a block in protein synthesis. The T domain is the most interesting, because it is responsible for delivering the catalytic domain into the cytoplasm of the target cell.

Endocytosis through binding of the B domain to the cell-surface receptor results in the sequestration of the toxin in the acidic interior of an endocytic vesicle. Exposure to the reducing environment inside the endocytic vesicle breaks the disulfide bond between the A and B domains, releasing the toxic A domain. At the same time, the acidic environment causes a massive conformational change in the T domain, which turns almost inside out, exposing the interior hydrophobic residues. Hydrophobic side chains, as we saw in Chapter 1 (section 1-9) are normally buried in soluble proteins, extensive exposed hydrophobic regions occurring only in the lipid interior of membranes (see section 1-11). Thus the exposure of hydrophobic residues of the diphtheria toxin T domain results in its insertion into the endosomal membrane, where it is thought to create a channel through which the toxic A domain is translocated into the cytoplasm (Figure 3-6).

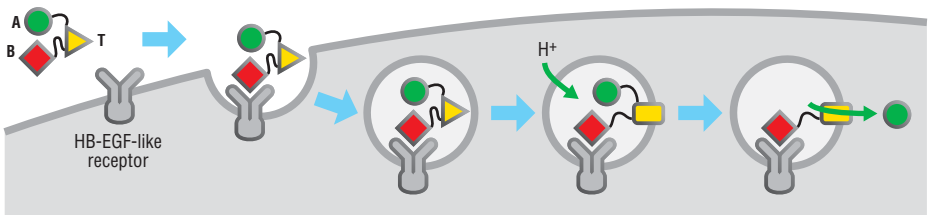

Figure 3-6 Schematic representation of the mechanism by which diphtheria toxin kills a cell The toxin has a receptor-binding domain (B), a membrane translocation domain (T) and a catalytic subunit (A) that kills cells by ADP-ribosylating a residue on the ribosomal protein EF-2. After receptor binding, the toxin enters the cell by endocytosis. In the low-pH environment of the endosome, a disulfide bond (not shown) in the toxin is reduced and a conformational change occurs that activates the membrane translocation domain, which is thought to release the active catalytic domain from the endosome. Weiss, M.S. *et al.*: *Biochemistry* 1995, **34**:773–781.

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3-4 Effector Ligands: Competitive Binding and Cooperativity

Protein function can be controlled by effector ligands that bind competitively to ligand-binding or active sites

One of the most important ways in which the activity of proteins is controlled is by binding regulatory molecules, termed **effector ligands** or effectors, that alter the activity of the protein with which they interact. Effectors can be as small as a proton (pH-induced conformational changes can be enormous, as in the case of the diphtheria toxin T domain discussed in the preceding section), or as large as another protein. In some instances this regulation can simply be inhibition through competitive binding with the normal ligand. This is a common mechanism for inhibiting enzymes, in which the effector ligand binds to the active site instead of substrate: many metabolic enzymes are feedback-inhibited in this way by their own product or by the product of an enzyme downstream from them in the same metabolic pathway (Figure 3-7). Feedback inhibition ensures that the activity of an enzyme is diminished when there is an overabundance of its product in the cell. An example is the control by the tripeptide glutathione (GSH) of its own biosynthesis. GSH is ubiquitous in mammalian and other living cells. It has several important functions, including protection against oxidative stress. It is synthesized from its constituent amino acids by the consecutive actions of two enzymes. The activity of the first enzyme is modulated by feedback inhibition of the end product, GSH, ensuring that the level of glutathione does not exceed necessary values.

Cooperative binding by effector ligands amplifies their effects

In the previous example, binding of one molecule of a competitive feedback inhibitor inhibits one molecule of a target enzyme. But many physiological responses need to be rapid and total, which is hard to achieve with a linear system. Amplification would allow a single regulatory molecule to shut down many copies of a target protein or pathway. Amplification can be achieved in either of two ways. One is by covalent modification of the protein, which we discuss later in this chapter. The other is by **cooperativity**. This is a phenomenon of universal importance in biological systems and is as versatile as it is widespread. At the metabolic level, an enzyme in one pathway can cooperate with another pathway by providing a component that can serve as a substrate, enzyme or regulator of that pathway. We are concerned here, on the other hand, with **cooperative binding**, cooperativity between binding sites for the same

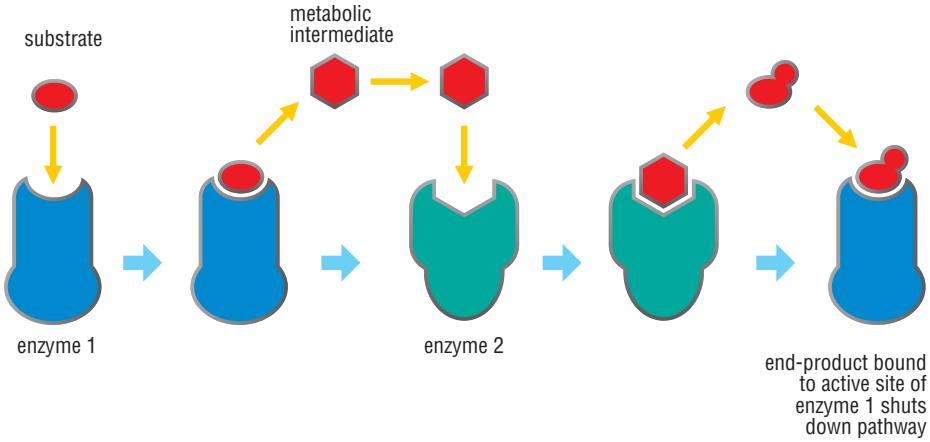

Figure 3-7 Competitive feedback inhibition
In this simple two-enzyme pathway, the end-product of the pathway acts as a competitive inhibitor of the first enzyme.

Definitions

cooperative binding: interaction between two sites on a protein such that the binding of a ligand to the first one affects the properties—usually binding or catalytic—of the second one.

cooperativity: interaction between two sites on a protein such that something that happens to the first one affects the properties of the second one.

effector ligand: a ligand that induces a change in the properties of a protein.

negative cooperativity: binding of one molecule of a ligand to a protein makes it more difficult for a second molecule of that ligand to bind at another site.

positive cooperativity: binding of one molecule of a ligand to a protein makes it easier for a second molecule of that ligand to bind at another site.

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ligand on a protein, in which binding of the ligand at one site affects the ease or otherwise of binding of ligand at the other site(s). This type of cooperativity depends on the ligand–protein interaction resulting in a measurable conformational change in other regions, close by or distant, of that protein. Cooperativity is only present in oligomeric proteins, where there are two or more subunits each with a binding site for the ligand.

Cooperativity can be positive or negative: **positive cooperativity** means that binding of one molecule of a ligand to a protein makes it easier for a second molecule of that ligand to bind; **negative cooperativity** means that binding of the second molecule is more difficult. Thus cooperativity can amplify either the activation of a protein or its inhibition. If a protein has, say, four identical subunits, each of which has a binding site for an effector ligand, positive cooperativity occurs if binding of the first effector molecule to one subunit makes binding easier (that is, increases the binding constant) for the second molecule of the effector to the second subunit. In turn, this binding facilitates binding of a third ligand molecule to the third subunit even more, and so on. This is illustrated for a dimer schematically in Figure 3-8. Such positive cooperativity means that activation (or inhibition) of all subunits can be achieved at a lower concentration of the activating or inhibiting ligand than would be the case if each subunit bound the effector independently and with equal affinity. In the extreme case of absolute positive cooperativity, only a few molecules of ligand might be sufficient to activate or inhibit the protein completely. Cooperative binding is also seen in proteins that are not enzymes. The oxygen-transport protein hemoglobin, for example, binds molecular oxygen at its four binding sites with positive cooperativity.

Negative cooperativity in enzymes usually involves the binding of substrates or cofactors, and in this case the binding site in question is the active site. In the extreme case of absolute negative cooperativity, a dimeric enzyme, for example, may never be found with cofactor or substrate bound to both subunits at the same time. For example, the bacterial enzyme ATP-citrate lyase shows strong negative cooperativity with respect to citrate binding. Non-enzymes can display this property as well. For example, the heart drug bepridil binds to the muscle protein troponin C with negative cooperativity. The physiological significance of absolute negative cooperativity is uncertain.

The phenomenon of cooperativity reflects an important consequence of the flexibility of proteins: binding of even a small molecule to a protein surface can induce structural changes at a distance from the binding site. In the case of oligomeric proteins, as we have seen, this can result in communication of a ligand-induced conformational change from one subunit to another. In the next section, we shall see how this ability of proteins to undergo long-range structural adjustments enables regulatory effector ligands to act by binding at sites remote from the normal ligand-binding or active site.

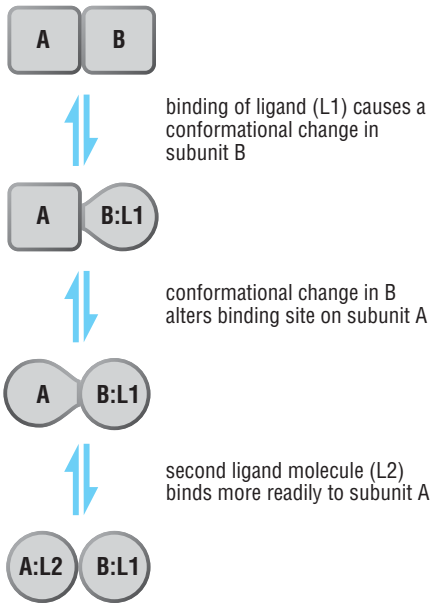

Figure 3-8 Cooperative ligand binding Shown here is a simple two-subunit protein displaying cooperative binding of a ligand L. The initial state of the protein (square) has a relatively low affinity for L. When the first molecule of L (L1) binds, it causes a conformational change in the subunit to which it is bound. But interactions between the subunits cause the unoccupied subunit to change its conformation as well, to one that is similar to the ligand-bound state (circle) and therefore has a higher affinity for L. Thus a lower concentration of L is required to bind to the second subunit, and the effect of L is amplified. If the protein is an enzyme, the effect of binding of the first ligand molecule may not only be to increase the affinity of the next subunit for ligand; its enzymatic activity may also be enhanced, increasing the amplifying effect.

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Effector molecules can cause conformational changes at distant sites

Because of the close packing of atoms in globular protein structures, even small changes in side-chain and main-chain positions at one site can be propagated through the tertiary structure of the molecule and cause conformational changes at a distant location in the protein. Indeed, the most common type of regulatory effector ligand is one that is different from the normal substrate or functional ligand for the protein and which binds at a site distinct from the enzyme's catalytic site or from the site through which the protein's function is mediated (in the case of a non-enzyme). In hetero-oligomeric enzymes, the regulatory site is often located on a different subunit from the active site.

There are two extreme models for the action of such regulators and evidence exists for each in different cases. In one, the binding of successive effector molecules causes a sequential series of conformational changes from the initial state to the final state (Figure 3-9a). The other model postulates a preexisting conformational equilibrium between a more active and a less active form of the protein. The effector can only bind to one of these forms, and thus its binding shifts the equilibrium in a concerted manner in favor of the bound form (Figure 3-9b). Because structural changes are produced in both models, this form of regulation is called **allostery** from the Greek for "another structure", and a protein that is regulated in this way is called an allosteric protein. A molecule that stabilizes the more active form is an **allosteric activator**; one that stabilizes the less active form is an **allosteric inhibitor**. When the effector ligand is another protein, the protein that is regulated can be, and often is, monomeric, as in the case of the regulation of the monomeric cyclin-dependent kinase Cdk2 by the protein cyclin A, as described later in this chapter. When the effector ligand is a small molecule, however, although allosteric regulation does not formally require cooperative binding, in practice the allosteric protein is nearly always oligomeric, and binding of the effector ligand is usually cooperative (see section 3-4).

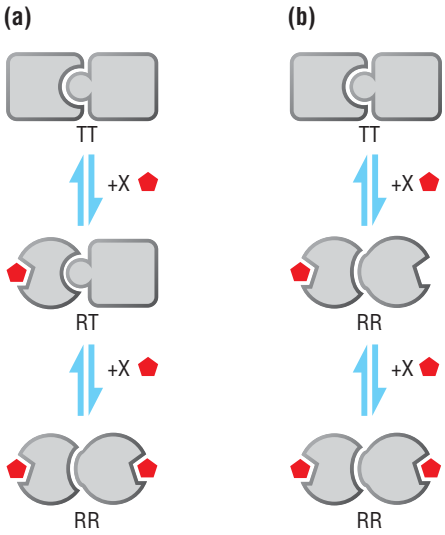

Figure 3-9 Two models of allosteric regulation **(a)** A sequential change in the conformation of subunits of a dimeric protein from a less active state (the "tense" state TT) to the more active state (the "relaxed" state RR) on binding of a regulatory ligand (X). **(b)** The case where the TT and RR states are in equilibrium and the regulatory ligand stabilizes one of them, shifting the equilibrium in favor of this form. To emphasize the conformational change, this diagram shows an asymmetrical protein; in reality, the subunits are usually identical.

ATCase is an allosteric enzyme with regulatory and active sites on different subunits

The allosteric enzyme aspartate transcarbamoylase (ATCase) is a hetero-oligomer made up of six catalytic and six regulatory subunits (Figure 3-10). ATCase catalyzes the formation of *N*-carbamoyl aspartate, an essential metabolite in the synthesis of pyrimidines, from carbamoyl phosphate and L-aspartate. The enzyme is allosterically inhibited by cytidine triphosphate (CTP), the end-product of pyrimidine nucleotide biosynthesis, and allosterically activated by adenosine triphosphate, the end-product of purine nucleotide biosynthesis. Thus, CTP is a feedback inhibitor and shuts down ATCase when pyrimidine levels are high; ATP activates the enzyme when purine levels are high and pyrimidines are needed to pair with them to make nucleic acids. Structural studies have shown that the enzyme exists in at least two states, with very different intersubunit contacts (Figure 3-10). The more compact state of the dodecamer (the tense or T state) is the less active form; it is stabilized by the binding of CTP to sites on the regulatory subunits. The more open arrangement (the relaxed or R state) is the more active enzyme; this structure is stabilized by binding of ATP, also to the regulatory subunits. Both these effectors bind cooperatively, as do the substrates, so there is great potential for amplification of the signals that regulate this enzyme. It is not surprising that the ATCase-catalyzed reaction is the major control step in pyrimidine biosynthesis.

Definitions

allosteric activator: a ligand that binds to a protein and induces a conformational change that increases the protein's activity.

allosteric inhibitor: a ligand that binds to a protein and induces a conformational change that decreases the protein's activity.

allostery: the property of being able to exist in two structural states of differing activity. The equilibrium between these states is modulated by ligand binding.

co-activator: a regulatory molecule that binds to a gene activator protein and assists its binding to DNA.

co-repressor: a regulatory molecule that binds to a gene repressor protein and assists its binding to DNA.

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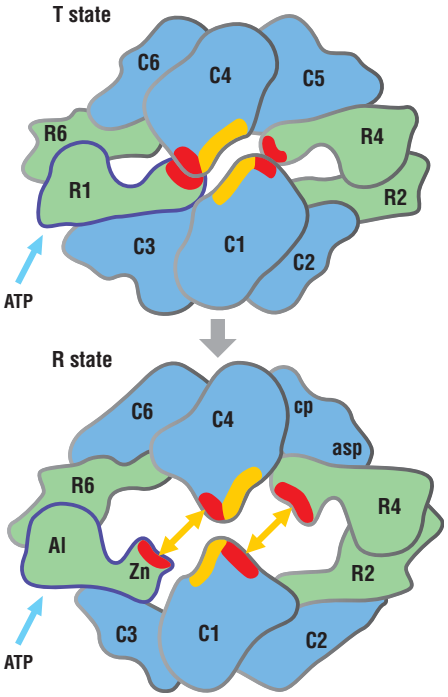

Figure 3-10 Ligand-induced conformational change activates aspartate transcarbamoylase
Binding of the allosteric activator ATP to its intersubunit binding sites on the regulatory subunits (that between R1, outlined in purple, and R6 is arrowed) of the T state of ATCase (top) causes a massive conformational change of the enzyme to the R state (bottom). In this state the structure of the enzyme is opened up, making the active sites on the catalytic subunits (C) accessible to substrate. Al and Zn in the lower diagram indicate the allosteric regions and the zinc-binding region, respectively; cp and asp indicate the binding sites for the substrates carbamoyl phosphate and aspartate, respectively. The red and yellow regions are the intersubunit interfaces that are disrupted by this allosteric transition.

Disruption of function does not necessarily mean that the active site or ligand-binding site has been disrupted

As we have seen, most proteins are like machines: they have moving parts. And like a machine, the introduction of a monkey wrench into any part of the protein can disrupt any activity that depends on the propagation of a conformational change. The monkey wrench can be a drug that binds directly to the active site or functional ligand-binding site, or a mutation at these sites. But drugs or mutations may also disrupt a protein's function by binding elsewhere and interfering with the conformational transitions necessary for function. For example, binding of anti-viral compounds to the rhinovirus coat protein blocks entry of the virus into host cells. But these drugs do not bind to the site on the virus that binds the cell-surface virus receptor; instead they bind to an unrelated site where they stabilize the structure of the coat protein, thereby preventing the structural rearrangements required for receptor-mediated virus entry into the cell.

The effects of both regulatory ligand binding and mutation are illustrated by ATCase. The T state can be stabilized by the binding of CTP to a site on the regulatory subunit. Exactly the same stabilizing effect can be achieved by mutation of tyrosine 77 to phenylalanine in the regulatory subunit. This mutation stabilizes the T state and thus shuts down catalytic activity, even though it is very far from the active site. Therefore, in the absence of structural information, one should not automatically assume that any mutation that disrupts a protein's function must be in the active site or that any molecule that inhibits function does so because it binds directly in the active site.

Binding of gene regulatory proteins to DNA is often controlled by ligand-induced conformational changes

DNA-binding proteins are not usually intended to interact with the genetic material all the time. Activators and repressors of gene expression are usually under the control of specific regulatory ligands, which may be small molecules, metal ions or proteins, whose binding determines whether or not the activator or repressor can bind to DNA. Such molecules are called **co-activators** and **co-repressors**, respectively. Expression of the gene encoding diphtheria toxin is under the control of a specific repressor, DtxR. Binding of DtxR to its operator sequence is controlled in turn by the concentration of Fe^{2+} in the bacterial cell. Iron acts as a co-repressor by binding to DtxR and inducing a conformational change that allows the helix-turn-helix DNA-recognition motif in the repressor to fit into the major groove of DNA. In the absence of bound iron, the repressor adopts a conformation in which DNA binding is sterically blocked (Figure 3-11).

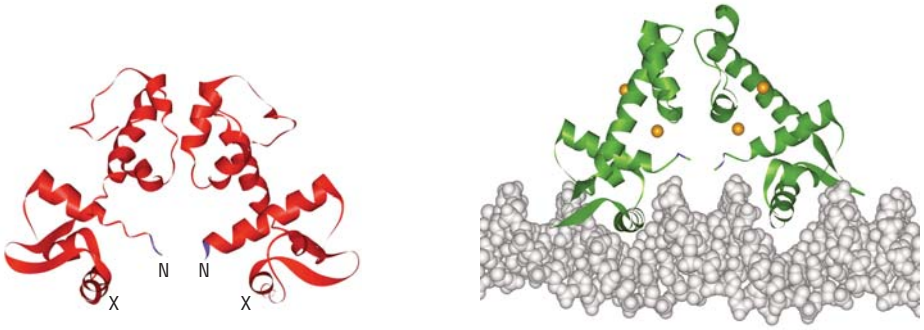

Figure 3-11 Iron binding regulates the repressor of the diphtheria toxin gene
Comparison of the structures of the aporepressor DtxR (red, left, PDB 1dpr) and the ternary complex (right) of repressor (green), metal ion (Fe^{2+}, orange) and DNA (grey) (PDB 1fst). Iron binding induces a conformational change that moves the recognition helices (X) in the DtxR dimer closer together, providing an optimal fit between these helices and the major groove of DNA. In addition, metal-ion binding changes the conformation of the amino terminus of the first turn of the amino-terminal helix (N) of each monomer. Without this conformational change, leucine 4 in this helix would clash with a phosphate group of the DNA backbone. Thus, DtxR only binds to DNA when metal ion is bound to the repressor.

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3-6 Protein Switches Based on Nucleotide Hydrolysis

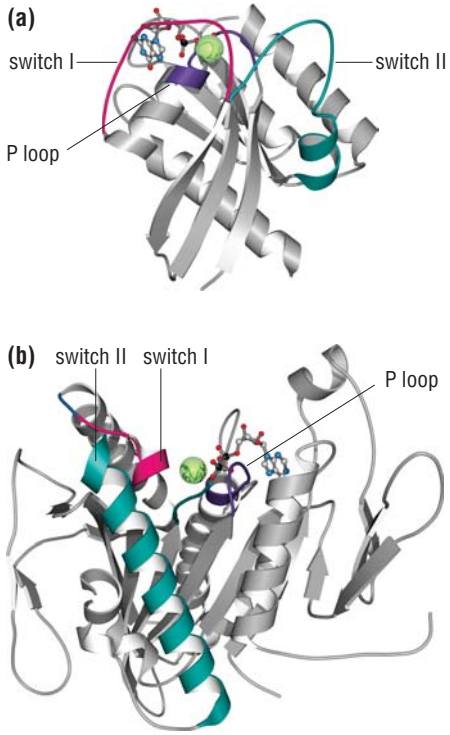

Figure 3-12 Structure of the core domains of a typical GTPase and an ATPase (a) Ribbon diagram of the minimal GTPase G domain, with the conserved sequence elements and the switch regions in different colors. The nucleoside diphosphate is shown in ball-and-stick representation and the bound Mg^{2+} ion as a green sphere. The switch I region is the pink loop that surrounds the ligand in this view. The switch II region is in turquoise and the P-loop in purple. (PDB 4q21) **(b)** Structure of the ATPase domain of the mitotic spindle kinesin Eg5. As in the GTPase, one molecule of Mg-ADP is located in the nucleotide-binding site. The equivalent switch I, switch II and P-loop regions are colored the same as for the GTPase. (PDB 1ii6)

Conformational changes driven by nucleotide binding and hydrolysis are the basis for switching and motor properties of proteins

Not every process in a living cell runs continuously. Many of them must cycle between "on" and "off" states in order to control cell growth and division and responses to extracellular signals. Signal transduction pathways such as those that operate in vision and hormone-based signaling, vesicular transport (which is often called protein or membrane trafficking), polypeptide chain elongation during protein synthesis, and actin- and tubulin-based motor functions are also examples of processes that must be switched on and off under precisely determined circumstances. This cycling is controlled by a special set of proteins that function as molecular switches. Although these proteins vary in structure and in the processes they control, they have a number of common features. The most important common element is the switching mechanism itself: most of these proteins undergo conformational changes induced by the difference between the triphosphate and diphosphate forms of a bound nucleotide. The conformational changes are such that completely different target proteins recognize the two bound states of the switch protein, providing a simple means of altering the output of a signal.

Most protein switches are enzymes that catalyze the hydrolysis of a nucleoside triphosphate to the diphosphate. Most often the nucleotide is guanosine triphosphate, GTP, and the switch protein is a GTPase that hydrolyzes it to GDP. GTPase switches (also commonly called **G proteins** or occasionally **guanine-nucleotide-binding proteins**) are one major class of switch proteins; they control the on/off states of most cellular processes, including sensory perception, intracellular transport, protein synthesis and cell growth and differentiation. The second major class of switch proteins is composed of those ATPases that are usually associated with motor protein complexes or transporters that move material into and out of cells and some organelles. Members of the third major class, the two-component response regulators, a group of switches thus far found only in microbes and plants, are composed of a histidine protein kinase and a second "response regulator" protein. They do not bind GTP or ATP in the same way as the nucleotide switch proteins discussed here, but use a covalently bound phosphate derived from the hydrolysis of ATP by the kinase to trigger a conformational change in the response regulator.

Hydrolyzable nucleotides are used to control many types of molecular switches because the energy derived from hydrolysis of the terminal phosphate of a GTP or ATP is large enough to make the conformational change in the switch effectively irreversible until another protein binds to the switch, displaces the diphosphate, and allows the triphosphate to bind again. Another reason is that using ATP or GTP can couple the switching process to the energy state of the cell and to the synthesis of DNA and RNA, both of which change the levels of nucleoside tri- and diphosphates.

All nucleotide switch proteins have some common structural and functional features

Nucleotide-dependent switch proteins are found in every kingdom of life. The ATPases and the GTPases constitute two protein families that are both characterized by a core domain that carries out the basic function of nucleotide binding and hydrolysis—called the G domain in the GTPases. The core domain structure is conserved within each family, but its secondary structure arrangement is very different in GTPases and ATPases. Remarkably, in spite of the lack of similarity between their protein folds, both the nucleotide-binding site and the switch mechanism are extremely similar for the two families. The standard fold of the G domain in

Definitions

G protein: a member of a large class of proteins with GTPase activity that act as molecular switches in many different cellular pathways, controlling processes such as sensory perception, intracellular transport, protein synthesis and cell growth and differentiation. They undergo a large conformational change when a bound GTP is hydrolyzed to GDP.

guanine-nucleotide-binding protein: see **G protein**.

P-loop: a conserved loop in GTPase- and ATPase-based nucleotide switch proteins that binds to phosphate groups in the bound nucleotide.

phosphate-binding loop: see **P-loop**.

switch I region: a conserved sequence motif in GTPase- and ATPase-based nucleotide switch proteins that, with the **switch II region**, binds the terminal gamma-phosphate in the triphosphate form of the bound nucleotide and undergoes a marked conformational change when the nucleotide is hydrolyzed.

switch II region: a conserved sequence motif in GTPase- and ATPase-based nucleotide switch proteins that, with the **switch I region**, binds the terminal gamma-phosphate in the triphosphate form of the bound nucleotide and undergoes a marked conformational change when the nucleotide is hydrolyzed.

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the GTPases consists of a mixed six-stranded beta sheet with five helices located on both sides (Figure 3-12a). There are three conserved features in nucleotide switches: the **P-loop**, and the **switch I** and **switch II** sequence motifs. The P-loop or **phosphate-binding loop** binds the alpha- and beta-phosphates from the phosphate tail of the nucleotide. Residues from the two switch motifs coordinate the terminal gamma-phosphate in the triphosphate form of the bound nucleotide. A Mg^{2+} ion, which is complexed with the bound nucleotide, is coordinated by the nucleotide phosphate groups and, in the triphosphate form of the switch, by residues from the switch I and II regions.

These regions usually contain four to five conserved sequence elements, which are lined up along the nucleotide-binding site. Additional important contribution to binding is made by the interactions of the nucleotide base with a sequence that has the motif N/TKXD (where X is any amino acid) and confers specificity for guanine. Specificity for guanine is due to the aspartate side chain in this motif, which forms a bifurcated hydrogen bond with the base, and to a main-chain interaction of an invariant alanine (from a short SAK motif), with the guanine oxygen.

Similar conserved sequence motifs are located in equivalent spatial positions in the switch ATPases, even though the core protein fold is completely different (Figure 3-12b). The adenine base interacts with a conserved RXRP or NP motif (equivalent to the N/TKXD in the GTPases) and alpha- and beta-phosphates are bound to an equivalent P-loop with the same consensus sequence GXXXXGKS/T. Furthermore, both switch I and switch II sequence motifs of GTPases (DX_nT and DXXG respectively) have their equivalents in ATPases: NXXSSR and DXXG, respectively.

The GTPases have been most extensively studied and most of the details of the switch mechanism have been determined for them, but the basic switch mechanism is the same for the ATPases. The trigger for the conformational change is most likely universal. The triphosphate-bound state, which is usually the "on" state of the switch, can be considered as "spring-loaded" because of the terminal phosphate group of the bound nucleotide, which makes a number of interactions with the two switch regions (Figure 3-13). It is the loss of this gamma-phosphate group on hydrolysis of GTP to GDP (or ATP to ADP) that provides the trigger for the conformational change.

Changes in conformation between the triphosphate- and diphosphate-bound states are confined primarily to the two switch regions. These regions usually show an increased flexibility relative to the rest of the protein when the structure of a nucleotide-dependent switch is determined. In the triphosphate-bound form, there are two hydrogen bonds from gamma-phosphate oxygens to the main-chain NH groups of the invariant threonine (or serine in the case of the ATPases) and glycine residues in the switch I and II regions, respectively (see Figure 3-13). The glycine is part of the conserved DXXG motif; the threonine (or serine) is from the conserved sequence involved in binding the Mg^{2+} ion that coordinates to the two terminal phosphate groups of the bound nucleotide. Release of the gamma-phosphate after hydrolysis allows the two switch regions to relax into their diphosphate-specific conformations because the bonds that the threonine/serine and glycine residues make with this phosphate are disrupted when it leaves. The extent of the conformational change that results from the rearrangement of the two switch regions is different for different proteins and involves additional structural elements in some of them.

Although the fold of the core domain is conserved, there are many insertions of other domains in individual GTPases. These domains have various functions, from interacting with other proteins to magnifying the conformational change on nucleotide hydrolysis.

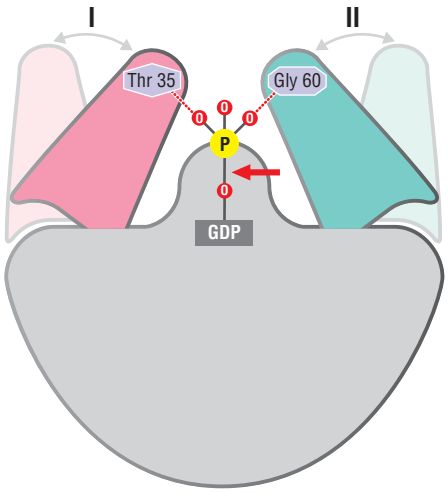

Figure 3-13 Schematic diagram of the universal switch mechanism of GTPases This picture illustrates the GTP-bound state where the switch I (pink) and switch II (turquoise) domains are bound to the gamma-phosphate via the main chain NH groups of the invariant threonine and glycine residues, in a spring-loaded mechanism. Release of the gamma-phosphate after GTP hydrolysis allows the switch regions to relax into a different conformation, as seen in Figure 3-12, which shows the diphosphate-bound forms. Adapted from Vetter, I.R. and Wittinghofer, A.: *Science* 2001, **294**:1299–1304.

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An animation showing the conformational changes in the switch regions of Ras can be found at: http://www.mpi-dortmund.mpg.de/departments/dep1/gtpase/ras_gtp_gdp.gif

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The switching cycle of nucleotide hydrolysis and exchange in G proteins is modulated by the binding of other proteins

All GTPase switches operate through conformational changes induced by a change in the form of the bound nucleotide, as we saw in the previous section; but this is only the core part of the switching mechanism. Which cellular process a particular G protein controls is determined by the interactions it makes with other proteins in its two conformational states. We will now consider the rest of the switching mechanism in detail in the case of the GTPases, exemplified by the small monomeric GTPase Ras, whose switch function helps to control cell growth and division.

The overall switching mechanism can be viewed as an on–off cycle in which the GTP-bound state is the "on" state and the GDP-bound state is "off". If we start with the switch in the off position, the gamma-phosphate of GTP is not present and the two switch regions are in the relaxed conformations characteristic of the diphosphate-bound state of the protein (see Figure 3-12a). GDP must now dissociate from the protein to allow GTP to bind. The normal rate of dissociation is often very slow, so most G proteins have various **guanine-nucleotide exchange factors** (**GEFs**) that bind to them and facilitate the release of GDP by inducing conformational changes that open up the binding site. A single G protein may be recognized by multiple GEFs, enabling one GTPase to serve as the focal point for integrating signals from different upstream pathways.

After GDP has been released and GTP binds, the protein conformation (that is, the arrangement of the switch I and switch II regions) changes to bind the gamma-phosphate (see Figure 3-13) and the switch is now in the on position. The altered conformation of the switch regions allows the G protein to interact with downstream effectors, activating various enzymes such as phosphatidylinositol-3-kinase and turning on various signaling and other pathways. Several different effectors may recognize the on state of the same G protein, allowing a single switch to control multiple cellular processes. The switch remains on as long as GTP remains bound to the GTPase.

Although GTPases are enzymes and catalyze the hydrolysis of GTP to GDP, they are not very efficient; their intrinsic rate of GTP hydrolysis is very slow. What determines the length of time the switch remains in the on state is the activity of various **GTPase-activating proteins** (**GAPs**) that bind to the GTP-bound conformation and stimulate the catalytic activity: in the case of Ras the binding of a GAP increases the GTPase activity by 100,000-fold (10^5). When GTP has been hydrolyzed, the switch I and switch II regions change to their relaxed conformations and the switch is back in the off state; the cycle is complete. Again, a single G protein can interact with multiple GAPs from different upstream signaling pathways. The on–off cycle is illustrated in Figure 3-14.

How the various GAP proteins facilitate GTP hydrolysis is not known in all cases, but at least some appear to function by stabilizing the transition state (see section 2-9). They insert an arginine side chain into the nucleotide-binding site of the GTPase to which they bind, and the positive charge on this side chain helps to stabilize the negative charge that builds up in the transition state for hydrolysis of the gamma-phosphate group of GTP.

The importance of proper regulation of G-protein signaling is exemplified by the Ras family of small GTPases. There are three human *RAS* genes, *H-*, *N-* and *K-RAS*, all of which code for very similar proteins of around 21 kDa molecular mass. They are post-translationally modified by the covalent attachment of lipophilic groups to the carboxy-terminal end. This modification is necessary for the Ras proteins' biological function as switches because it targets them to the

Definitions

GTPase-activating protein (GAP): a protein that accelerates the intrinsic GTPase activity of switch GTPases.

guanine-nucleotide exchange factor (GEF): a protein that facilitates exchange of GDP for GTP in switch GTPases.

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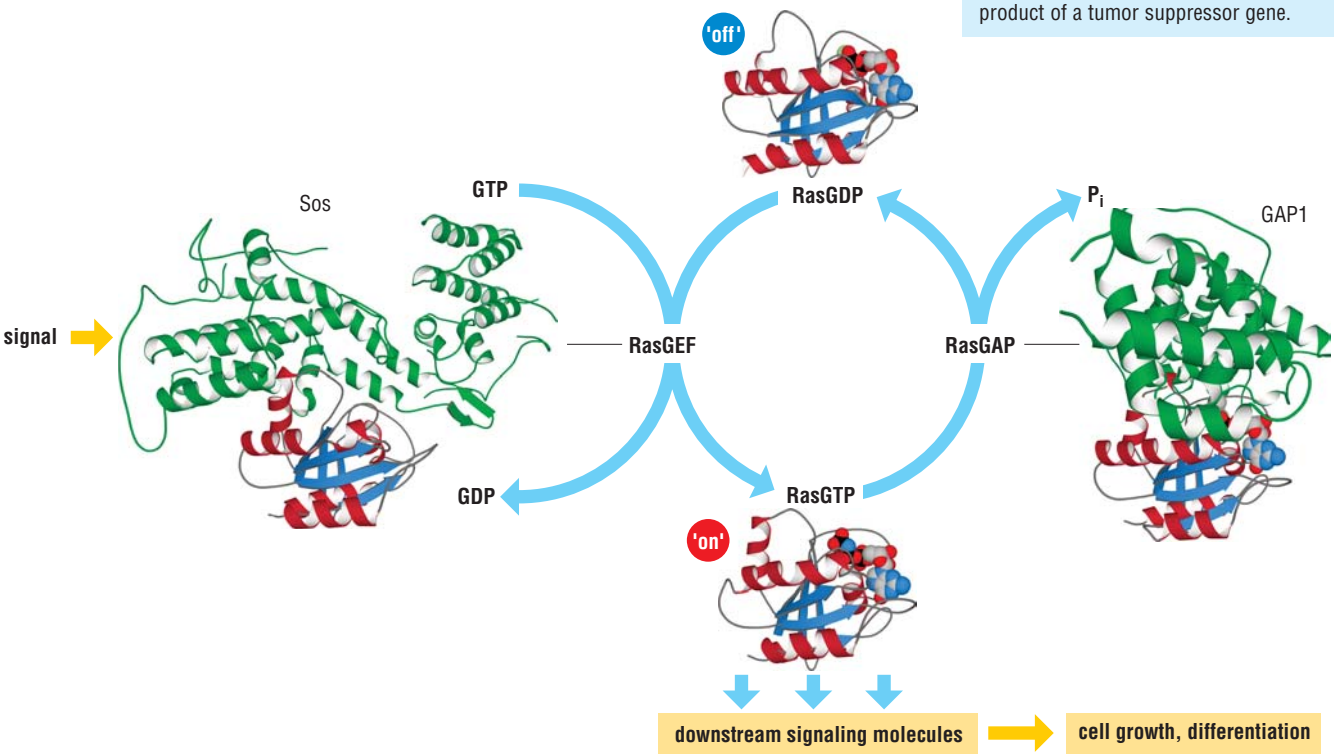
plasma membrane, where their interacting protein partners are found (see Figure 3-4). Interestingly, the pathways controlled by Ras seem to differ in mouse, humans and yeast. Consequently, mutations in the corresponding genes lead to different phenotypes in different organisms. This is also true for some other signal transduction pathways, and it complicates the generalization of data obtained from model organisms such as the mouse.

The lifetime of the signal transduced by Ras is determined by the lifetime of the GTP-bound state. If it is artificially prolonged, the biological response may be unregulated and lead to drastic consequences in the cell. The gene coding for Ras was originally discovered as an oncogene of rodent tumor viruses, the gene responsible for their ability to cause cancer in animals. Mutant forms of the human *RAS* genes that produce a protein with a prolonged on state are found in up to 30% of human tumors. This activated Ras protein is the result of point mutations at amino acids 12, 13 or 61, the biochemical consequence of which is to reduce the rate of hydrolysis of GTP. This leaves Ras in the on state too long, leading to uncontrolled cell growth and proliferation. As many other genes involved in the Ras signal transduction pathway are also found as oncogenes in human or animal tumors, Ras itself and the Ras pathway is considered to be a prime target for anti-tumor therapy.

Figure 3-14 The switching cycle of the GTPases involves interactions with proteins that facilitate binding of GTP and stimulation of GTPase activity The cycle is illustrated here for the small G protein Ras, but the principles are the same for other GTPase switches such as protein synthesis elongation factors and the heterotrimeric G proteins, which are discussed later in this chapter. GTP-binding proteins function as molecular switches by cycling between GDP-bound "off" and GTP-bound "on" states. Exchange of the bound GDP for GTP is facilitated by guanine-nucleotide exchange factors (GEFs) whose binding to the GTPase increases the dissociation rate of the nucleotide by several orders of magnitude. Ras can be switched on by several GEFs, the most important of which are Sos (illustrated here), Ras-GRF and Ras-GRP. Sos is part of the pathway that conveys signals from an activated cell-surface receptor to Ras. In the activated GTP-bound state Ras interacts with and activates several target proteins involved in intracellular signaling pathways. Ras is switched off by hydrolysis of the bound GTP. This reaction is facilitated by the action of specific GTPase-activating proteins (GAPs), the best studied of which are GAP1 (illustrated here), p120GAP and neurofibromin, the product of a tumor suppressor gene.



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3-8 GTPase Switches: Signal Relay by Heterotrimeric GTPases

Heterotrimeric G proteins relay and amplify extracellular signals from a receptor to an intracellular signaling pathway

Most GTPases are either small monomeric proteins like Ras or much larger, heterotrimeric molecules, **heterotrimeric G proteins**, which are composed of an α subunit, whose core polypeptide chain fold is the same as the canonical G-domain fold, and β and γ subunits whose folds bear no relationship to that of the GTPases (Figure 3-15). The α subunit has an extra helical domain compared with the canonical G domain, and this partially projects into the catalytic site. The β and γ subunits are tightly associated with one another, partly by means of a coiled-coil interaction. Heterotrimeric G proteins are associated with the cytoplasmic surface of the cell membrane in complexes with so-called G-protein-coupled receptors (GPCRs), integral membrane proteins with seven transmembrane alpha helices. Affinity of heterotrimeric G proteins for the membrane is aided by prenylation of the β and γ subunits. In general, GPCRs are activated as a result of the binding of specific extracellular ligands to their extracellular or transmembrane domains. Conformational changes in the receptor induced by ligand binding are relayed through the transmembrane domain to the cytoplasmic portion, allowing productive coupling of the receptor with a heterotrimeric G protein, which transduces the signal and relays it onwards by means of its switching function.

The switching cycle of the heterotrimeric G proteins resembles that of the small monomeric GTPases with additional features imparted by the presence of the β and γ subunits. Heterotrimeric G proteins bind to the cytoplasmic domains of GPCRs in their GDP-bound "off" states. When activated by binding of their extracellular ligand, these receptors act as guanine-nucleotide exchange factors (GEFs) for their partner heterotrimeric G proteins, thus triggering GDP dissociation and the GTPase switching cycle. The βγ complex acts as part of the nucleotide-exchange mechanism. In the absence of βγ, α does not bind to GPCR. In addition, βγ does not bind to α when α is in its GTP form. Consequently, βγ acts to guarantee that GPCR only catalyzes replacement of GDP by GTP and not the other way around.

When GDP is released and GTP binds, the heterotrimeric G protein dissociates from the GPCR and the β and γ subunits dissociate as a heterodimer from the α subunit. Both the free GTP-bound α subunit and the βγ heterodimer can now bind to and stimulate their own respective downstream effectors, which are generally ion channels and enzymes. Hydrolysis of GTP in the active site of the free α subunit then results in a conformational change in the switch I and switch II regions, which restores association of α to the βγ heterodimer and causes the reassembled heterotrimeric G protein to rebind to the GPCR, completing the cycle.

Just as GAP proteins switch the state of small monomeric GTPases from GTP-bound to GDP-bound by increasing the GTPase catalytic rate, so-called **regulator of G-protein signaling proteins (RGS proteins)** are responsible for the rapid turnoff of GPCR signaling pathways by functioning as activators of the heterotrimeric G protein GTPase. Structural and mutational analyses have characterized the interaction of the RGS domain of these proteins with Gα in detail. Unlike RasGAPs, the RGS proteins do not directly contribute an arginine residue or any other catalytic residue to the active site of the α subunit to assist GTP hydrolysis. In fact, the α subunit of most heterotrimeric G proteins has a "built-in" arginine residue in the extra helical domain that projects into the catalytic site. RGS proteins probably exert their GAP activity mainly through binding to the switch regions, reducing their flexibility and stabilizing the transition state for hydrolysis in that way. More than 20 different RGS proteins have been isolated, and there are indications that particular RGS proteins regulate particular GPCR signaling pathways. This specificity is probably created by a combination of cell-type-specific

Definitions

heterotrimeric G protein: a GTPase switch protein composed of three different subunits, an α subunit with GTPase activity, and associated β and γ subunits, found associated with the cytoplasmic tails of G-protein-coupled receptors, where it acts to relay signals from the receptor to downstream targets. Exchange of bound GDP for GTP on the α subunit causes dissociation of the heterotrimer into a free α subunit and a βγ heterodimer; hydrolysis of the bound GTP causes reassociation of the subunits.

RGS protein: regulator of G-protein signaling protein; protein that binds to the free GTP-bound α subunit of a **heterotrimeric G protein** and stimulates its GTPase activity.

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expression, tissue distribution, intracellular localization, post-translational modifications, and domains other than the RGS domain that link these RGS proteins to other signaling pathways. Many heterotrimeric G proteins operate as complexes that include the RGS protein and the GPCR. This association causes rates of GTP hydrolysis and nucleotide exchange (the deactivation and activation rates, respectively, for the G protein) to become rapid and tightly coupled as long as the ligand for the receptor is present.

GPCRs, which are characterized by seven membrane-spanning alpha helices (Figure 3-15), are the most numerous receptors in all eukaryotic genomes (1–5% of the total number of genes). They transduce extracellular signals as varied as light, odorants, nucleotides, nucleosides, peptide hormones, lipids, and proteins. Rhodopsin, the primary photoreceptor protein in vision, is a prototypical GPCR that contains 11-*cis*-retinal as an intrinsic chromophore. The light-induced *cis–trans* isomerization of this ligand is the primary signaling event in the visual process. It is followed by slower and incompletely defined structural rearrangements leading to an "active state" intermediate of rhodopsin that signals via the heterotrimeric G protein transducin.

As well as relaying the signal onwards, heterotrimeric G proteins can also be one means by which it is amplified. It has been estimated that one rhodopsin molecule activated by a single photon of light activates around 500 molecules of transducin. Each of these activates a molecule of its target phosphodiesterase.

There are at least eight families of GPCRs that show no sequence similarities to each other and that have quite different ligand-binding domains and cytoplasmic domains, but which nevertheless activate a similar set of heterotrimeric G proteins. Homo- and heterodimerization of GPCRs seems to be the rule, and in some cases an absolute requirement, for activation. It has been estimated that there are about 100 "orphan" GPCRs in the human genome for which ligands have not yet been found. Mutations of GPCRs are responsible for a wide range of genetic diseases, including some hereditary forms of blindness. The importance of GPCRs in physiological processes is further illustrated by the fact that they are the targets of the majority of therapeutic drugs and drugs of abuse.

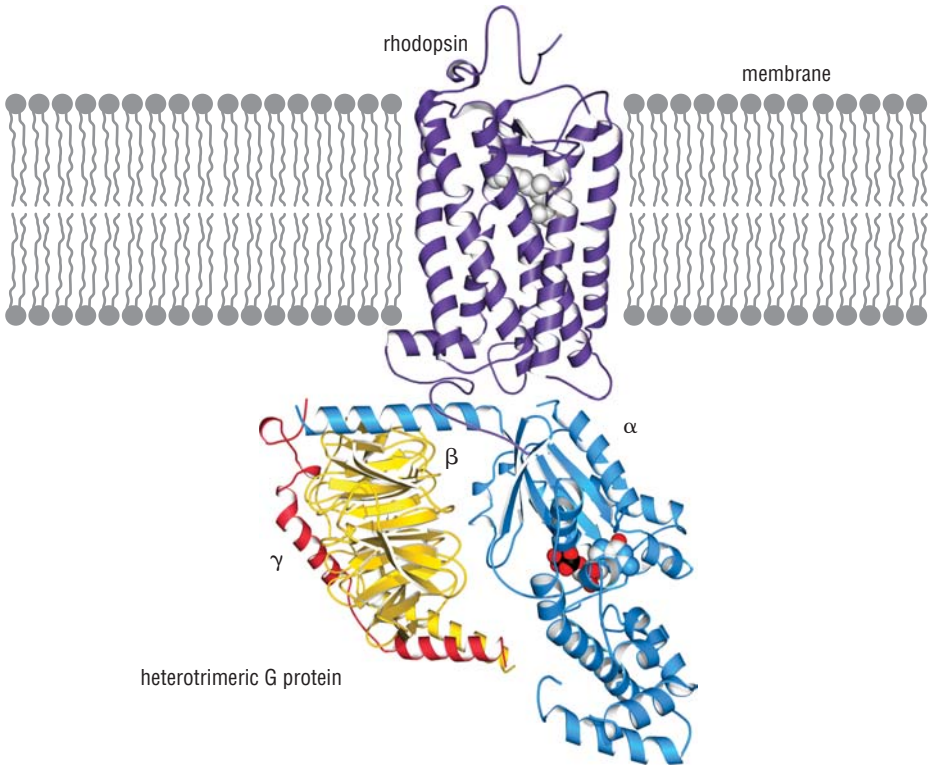

Figure 3-15 Hypothetical model of a heterotrimeric G protein in a complex with its G-protein-coupled receptor The GTPase subunit is shown in blue, the β subunit, which consists of a series of tandem WD40 domains, in yellow, and the γ subunit, which binds to the β subunit by a coiled-coil interaction, in red. The GPCR, shown here as the photoreceptor rhodopsin, is in purple embedded in a schematic of a membrane. A prenylation site on the α subunit that targets the complex to the membrane is not shown. GDP bound to the α subunit is shown in space-filling representation. Rhodopsin is activated by the photoisomerization of 11-*cis*-retinal (grey) bound to the protein. Rhodopsin has quite a short cytoplasmic tail but many other GPCRs have large cytoplasmic domains and additional extracellular domains to which extracellular ligands bind. Because the actual structure of the complex of any heterotrimeric G protein with its receptor is not known, the precise interactions between the components should not be inferred from this model. Adapted from Hamm, H.E. and Gilchrist, A.: *Curr. Opin. Cell Biol.* 1996, **8**:189–196.

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EF-Tu is activated by binding to the ribosome, which thereby signals it to release its bound tRNA

One of the central protein components in the machinery of protein synthesis is a guanine-nucleotide-dependent switch. This molecule, called elongation factor Tu or EF-Tu in prokaryotes (the analogous factor in eukaryotes is called EF-1), consists of a core domain that has the canonical switch GTPase fold, and has two other domains that help bind a molecule of transfer RNA. Although there are many different tRNA molecules required for protein synthesis, they have the same overall structure and all can bind to the same EF-Tu. tRNA only binds to the GTP-bound state of EF-Tu; GTP hydrolysis causes dissociation of the complex.

EF-Tu performs a key function in protein synthesis: in its GTP-bound form it escorts aminoacyl-tRNAs coming into the ribosome, where its function is to facilitate codon–anticodon interactions and check their fit. If the fit is correct, this causes conformational changes in the ribosome that stabilize tRNA binding and trigger GTP hydrolysis by EF-Tu. On this, the elongation factor dissociates from the tRNA and leaves the ribosome, leaving the tRNA behind to deliver its amino acid to the growing polypeptide chain (Figure 3-16). If the pairing is incorrect, however, the codon–anticodon interaction is weaker, and this permits the aminoacyl-tRNA–EF-Tu complex to dissociate before hydrolysis of GTP and release of the tRNA can occur. Thus EF-Tu increases the ratio of correct to incorrect amino acids incorporated by providing a short delay between codon–anticodon base pairing and amino-acid incorporation.

tRNA release is driven by the same nucleotide-dependent switch mechanism that operates in the small monomeric and heterotrimeric G proteins. GTP hydrolysis causes a change in the conformations of the two switch regions, which in turn is transmitted to an alpha helix linked to one of them. The helix is part of the interface between the GTPase domain and the other two domains of EF-Tu, and when it moves the entire interface rearranges, which in turn causes a change in the conformations and relative positions of all three domains. The domains, which were closely associated with the tRNA and with each other in the GTP-bound state, now swing apart, one of them moving by almost 40 Å. This opening up of the protein structure dissociates the elongation factor from the bound tRNA.

Thus, in EF-Tu switching it is the correct pairing of the bound tRNA anticodon with the codon in the mRNA on the ribosome that acts as a GAP to promote the GTPase activity. By analogy, one expects to find the equivalent of a GEF to promote exchange of GTP for the bound GDP that remains on EF-Tu after hydrolysis. This function is provided by another protein, elongation factor Ts. When EF-Ts binds to the GDP-bound state of EF-Tu, the nucleoside diphosphate is released and GTP is able to bind. Binding of tRNA to the GTP-bound form completes the cycle (Figure 3-16). EF-Tu resembles the heterotrimeric G proteins in that GTP hydrolysis triggers a large-scale conformational change that leads to a loss of protein–protein interactions, but here the interactions are among domains in the same polypeptide chain rather than between non-identical subunits of an oligomeric protein.

The importance of EF-Tu switching in protein synthesis is underscored by the fact that dozens of antibiotics act by binding to EF-Tu and interfering with its function (although the human homolog EF-1 is similar in structure and function, it differs enough to be unaffected by these molecules). For instance, aurodox is a member of the family of kirromycin antibiotics, which inhibit protein biosynthesis by binding to EF-Tu. When aurodox is bound, the GTP-bound conformation of EF-Tu is observed, even when GDP is bound to the nucleotide-binding site.

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This suggests that aurodox fixes EF-Tu on the ribosome by locking it in its GTP form. Certain mutations in bacterial EF-Tu genes confer resistance to kirromycin by producing a protein that cannot interact with the antibiotic.

EF-Tu is not the only GTPase switch involved in translation. Another elongation factor, EF-G, is involved at a later step, in the translocation of the mRNA through the ribosome so that it is ready to accept the next aminoacyl-tRNA.

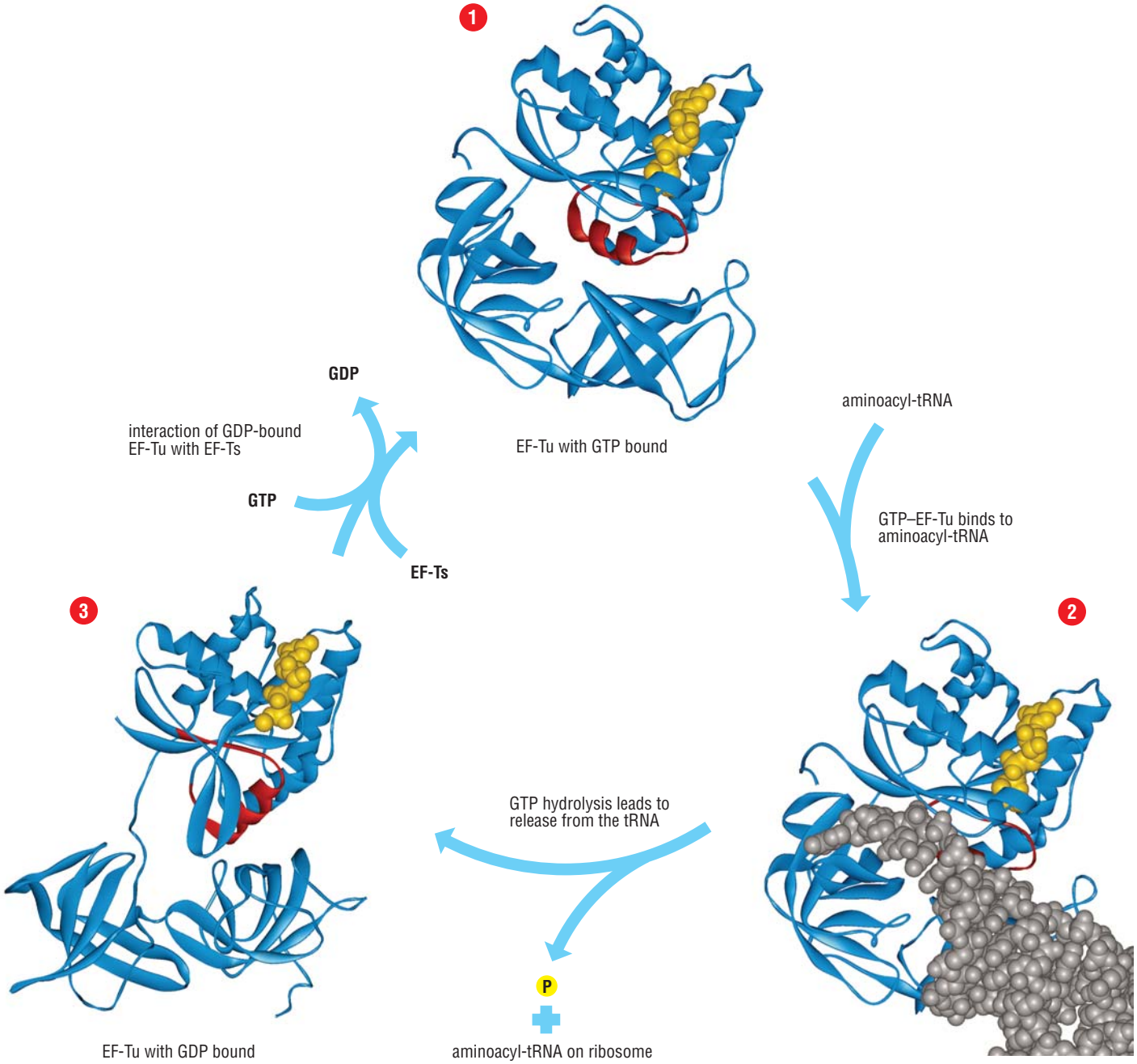

Figure 3-16 The switching cycle of the elongation factor EF-Tu delivers aminoacyl-tRNAs to the ribosome EF-Tu in its GTP-bound form (structure 1 at top; GTP shown in yellow bound to the GTPase domain) binds to an aminoacyl-tRNA (structure 2; the tRNA is in grey). This complex binds to the mRNA codon displayed in the A site on the small subunit of the ribosome through pairing of the codon with the anticodon on the tRNA (not shown). Correct pairing triggers GTP hydrolysis by EF-Tu, causing a conformational change that dissociates the factor from the tRNA molecule, which can now deliver its amino acid to the growing polypeptide chain. Generally, only those tRNAs with the correct anticodon can remain paired to the mRNA long enough to be added to the chain. The GDP form of EF-Tu (structure 3; GDP shown in yellow) released from the ribosome then interacts with EF-Ts, its guanine-nucleotide exchange factor (not shown), to facilitate exchange of GDP for GTP. The GTP-bound form of EF-Tu (1) can then restart the cycle. Shown in red is a helical segment in the GTPase domain that undergoes a significant conformational change between the GTP-bound (1) and GDP-bound (3) forms of the enzyme. The two forms also differ markedly in the relative arrangements of their domains.

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Myosin and kinesin are ATP-dependent nucleotide switches that move along actin filaments and microtubules respectively

Many processes in living organisms and cells require movement. Directed transport of macromolecules, vesicles, organelles and chromosomes within the cytoplasm depends on motors that drive such transport. Bacterial swimming and the movement generated by muscles in higher organisms are examples of processes that also require motor power. Most biological motors move unidirectionally along protein polymers such as actin or microtubules; these polymers can be considered the rails along which protein motor "engines" move cargo; the same sort of machinery is used to slide the actin and myosin filaments past one another in muscle contraction. Defective molecular transport can result in developmental defects as well as cardiovascular and neuronal diseases.

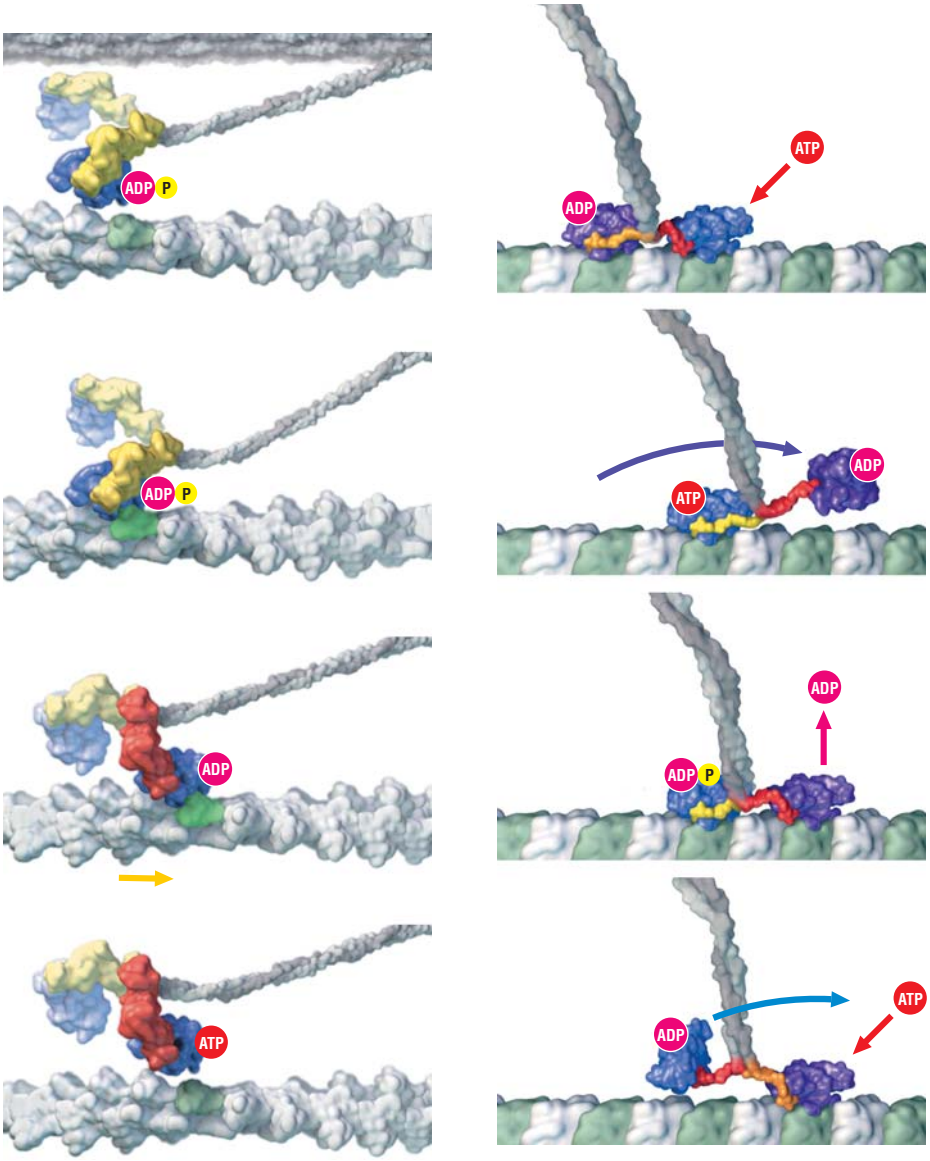

Figure 3-17 Models for the motor actions of muscle myosin and kinesin Left panel, the sequential conformational changes in muscle myosin result in a rowing "stroke" that moves an actin filament. Muscle myosin is a dimer of two identical motor heads which are anchored in the thick myosin filament (partially visible at the upper edge of the frame) by a coiled-coil tail. At the start of the cycle (top frame of the sequence) ADP and inorganic phosphate (P_i) are bound to the heads as a result of hydrolysis of a bound ATP by the intrinsic ATPase activity of the catalytic cores (blue). The lever-arms that eventually cause the movement are shown in yellow in their "prestroke" conformation. In this conformation the catalytic core binds weakly to actin (bottom of frame). The second frame shows one myosin head docking onto a specific binding site (green) on the actin thin filament (pale grey). (The two myosin heads act independently, and only one attaches to actin at a time.) As shown in the third frame, on actin docking P_i is released from the active site, and there is a conformational change in the head that causes the lever arm to swing to its "poststroke" ADP-bound position (red) while the head remains bound to the actin. This moves the actin filament by approximately 100 Å in the direction shown by the arrow. After completing the stroke, ADP dissociates and ATP binds to the active site and undergoes hydrolysis, reverting the catalytic core domain to its weak-binding actin state, and bringing the lever arm back to its prestroke state. Right panel, kinesin "walks" along a microtubule, its two head domains moving in front of each other in turn. The coiled-coil tail of the kinesin (grey) leads to the attached cargo (not shown). The catalytic heads are shown in blue and purple and the microtubule in green and white. Movement is generated by conformational changes in the linker regions that join the heads to the tail and are colored here according to their conformational state: red, not bound to microtubule; orange, partially bound to microtubule; yellow, tightly bound to microtubule. The direction in which the kinesin will move is shown by the arrows. At the beginning of the movement cycle, the "trailing" head has ADP bound and the "leading" head is empty and neither linker is docked tightly to the microtubule. As shown in the first two frames, when ATP binds to the leading head, its linker (red in the top frame and yellow in the second frame) adopts a conformation that as well as docking it firmly to the microtubule reverses its position and thus throws the trailing head forward by about 160 Å (arrow) towards the next binding site on the microtubule (second frame). This head docks onto the binding site (third frame), which moves the attached cargo forward 80 Å. Binding also accelerates the release of ADP from this head, and during this time the ATP on the other head is hydrolyzed to ADP-P_i (third frame). After ADP dissociates from the new leading head ATP binds in its turn, causing the linker to zipper onto the core (partially docked linker in orange). The new trailing head, which has released its phosphate and detached its neck linker (red) from the core, is being thrown forward. Original illustration kindly provided by Graham Johnson. From Vale, R.D. and Milligan, R.A.: *Science* 2000, **288**:88–95, with permission.

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Nearly all molecular motors share a common feature: a core ATPase domain that binds and hydrolyzes ATP, and in doing so switches between different conformations in a process that is similar to the GTPase molecular switches but which is carried out with a very different protein scaffold. Attached to the core domain are smaller transmission or converter domains that read out the nucleotide-dependent conformation of the core and respond by conformational changes. In turn, these domains relay the conformational change into larger motions by altering the positions of an amplifier region, very often a lever-arm or coiled coil. Large changes in the position of the amplifier in relation to an attached protein are what finally cause very large movements of proteins (Figure 3-17).

Although the overall folds of the ATPase domains of motors and the GTPase domains of G proteins are different, there are striking similarities in the local architecture around the bound nucleotide and in the mechanism of switching. Motor ATPases have switch I and switch II regions that correspond almost exactly to those in the G proteins, and these regions are involved in binding the gamma-phosphate of ATP, just as in the GTPases (Figure 3-18). When ATP is hydrolyzed by the intrinsic ATPase activity of the motor, the switch I and II regions relax into their ADP-bound conformational states, which starts the relay of conformational changes that are ultimately propagated into large movements of attached proteins and domains.

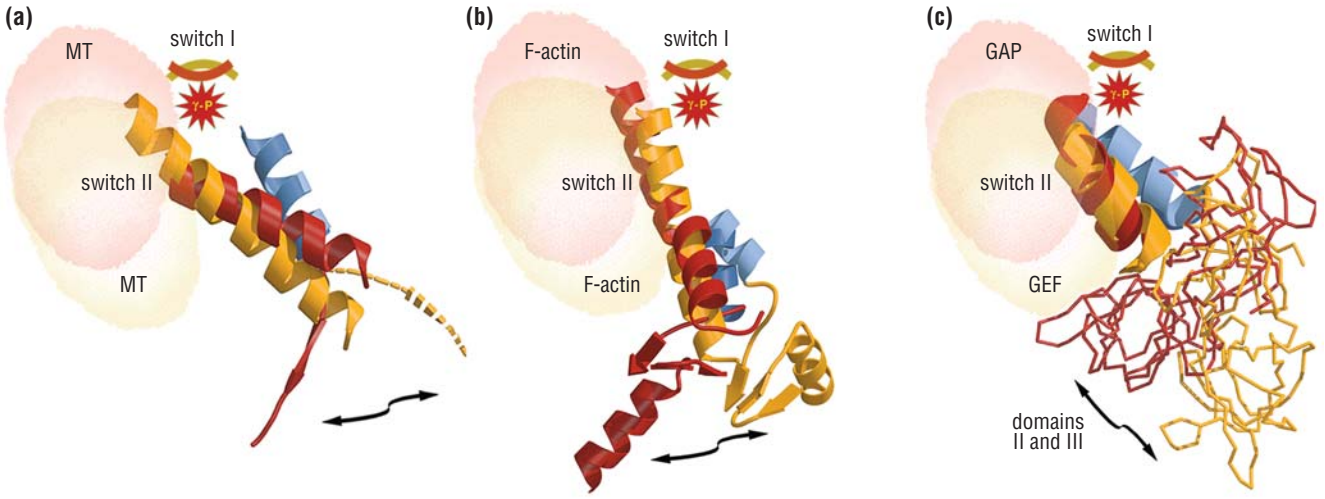

Figure 3-18 Structural and functional similarity between different families of molecular switches In all these graphics, the position of the switch II helix is shown in yellow for the nucleoside diphosphate-bound protein, and in red for the triphosphate-bound conformation. The switch I region is shown schematically with the gamma-phosphate of the nucleotide depicted as a red star. Specific macromolecular partners interact with the switch regions in these two states and are shown as surfaces of the same color. The carboxy-terminal helix of the ATPase or GTPase domain, to which the mechanical elements of molecular motors or additional domains of G proteins are attached, is shown in blue. **(a)** The switch II region of the motor protein kinesin. The nucleotide-driven conformational switching in the kinesin switch II region results in large force-generating rearrangements of the neck domain (see Figure 3-17; not shown here) and movement of kinesin along a microtubule (MT). **(b)** The same mechanism of switching in myosin converts small conformational changes in the nucleotide-binding site into larger movements of the lever-arm helix (see Figure 3-17), part of which is shown here at the bottom of the graphic (ADP state, PDB 2mys; ATP state, PDB 1br1). **(c)** An identical molecular mechanism is used by G proteins for their domain rearrangements and for controlling affinity for their specific macromolecular partners—GEFs, GAPs and effectors. As well as the switch II helix, domains II and III of EF-Tu are shown in GDP state (PDB 1tui) and the GTP state (PDB 1eft). Graphic kindly provided by E.P. Sablin. From Sablin, E.P. and Fletterick, R.J.: *Curr. Opin. Struct. Biol.* 2001, **11**:716–724, with permission.

looking under the hood of molecular motor proteins.
Science 2000, **288**:88–95.

A movie of kinesin moving may be found at:
http://valelab.ucsf.edu/images/mov-procmotconvkinrev5.mov

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3-11 Regulation by Degradation

Protein function can be controlled by protein lifetime

Proteins not only carry signals that determine their location, they also carry signals that determine their lifetime. Different proteins can have widely differing half-lives within the cell—anywhere from a few minutes to many days—and this time depends not only on the stability of the protein's structure but also on specific cellular machinery for degradation, which in some cases recognizes specific sequence and structural features. Stability in the extracellular environment is also variable, and depends on the intrinsic stability of the protein fold and the presence or absence of both relatively nonspecific and specific proteases. The shortest-lived proteins are usually those that are important in controlling cellular processes, such as enzymes that catalyze rate-determining steps in metabolic pathways or proteins such as cyclins that regulate cell growth and division. Rapid degradation of such proteins makes it possible for their concentrations to be changed quickly in response to environmental stimuli.

Protein degradation in cells is accomplished by machinery that tags both misfolded and folded proteins for specific proteolysis and destroys them. This mechanism depends in part on intrinsic protein stability at physiological temperature. One measure of the intrinsic stability of a protein is its resistance to thermal unfolding. Although there are microorganisms that survive at very high temperatures (so-called thermophilic organisms)—and in which, presumably, all proteins are fairly thermostable—very stable proteins are also found in the cytosol of mesophilic organisms (that is, organisms that live at normal temperatures). There appears to be no correlation between the type of protein fold and the intrinsic stability of a protein; rather, it is the specific sequence that determines stability, through the presence or absence of stabilizing interactions between side chains. Comparison of the structures of thermostable proteins with their mesophilic counterparts shows that many different stabilizing factors can be employed, mostly involving electrostatic interactions of various sorts. As the net free energy of stabilization of most folded proteins is relatively small, about 21–42 kJ/mole, only a few additional interactions can make a large difference in thermal stability.

Temperature-sensitive mutants, which are widely used in genetic research, are mutant organisms that produce a mutated protein that has reduced stability at physiological temperatures. These mutant proteins are more susceptible than the normal forms to degradation by non-specific proteases, and when such mutations occur in humans they can lead to disorders caused by insufficient amounts of the active protein.

Proteins are targeted to proteasomes for degradation

The proteolytic machinery responsible for targeted protein degradation in cells is a giant multiprotein assembly called the **proteasome** (Figure 3-19). In eukaryotes, proteins carrying an appropriate signal for destruction are recognized by an enzyme that then further tags the protein for degradation in the proteasome. The tag consists of many molecules of a small

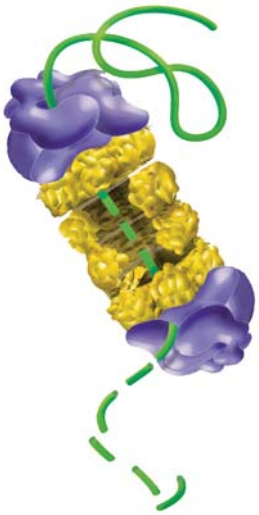

Figure 3-19 The eukaryotic proteasome Proteins targeted for destruction (green) are fed into the multiprotein complex called the proteasome. In prokaryotes, these machines of destruction consist simply of a tunnel-like enzymatic core; in eukaryotes they have an additional cap (here shown in purple) at either or both ends. The core is formed by four stacked rings surrounding a central channel that acts as a degradation chamber. The caps recognize and bind to proteins targeted by the cell for destruction. On entry into the proteasome, proteins are unfolded in a process that uses the energy released by ATP hydrolysis and injected into the central core, where they are enzymatically degraded into small fragments. Graphic kindly provided by US Department of Energy Genomes to Life Program, http://doegenomestolife.org.

Definitions

proteasome: a multiprotein complex that degrades ubiquitinated proteins into short peptides.

stress-response proteins: proteins whose synthesis is induced when cells are subjected to environmental stress, such as heat.

temperature-sensitive mutants: organisms containing a genetic mutation that makes the resulting protein sensitive to slightly elevated temperatures. The temperature at which the mutant protein unfolds is called the restrictive temperature. The term is also used for the protein itself.

ubiquitin: a small protein that when attached to other proteins (**ubiquitination**), targets them for degradation to the **proteasome**. Sometimes ubiquitin tagging targets a protein to other fates such as endocytosis.

ubiquitination: the attachment of ubiquitin to a protein.

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protein, **ubiquitin**, which are attached to the protein to be degraded and which are recognized by the cap. **Ubiquitination** is carried out by a multi-enzyme pathway that starts by recognition of an exposed lysine near the amino terminus of the target protein. Once one ubiquitin molecule is covalently attached to this lysine, by enzymes called ubiquitin ligases, additional ubiquitins are attached to the first one, producing a polyubiquitin chain. Polyubiquitinated proteins are the primary substrates for the proteasome, which in an ATP-dependent process degrades these proteins to short peptides (Figure 3-20). In prokaryotes, a homolog of the proteasome acts without such a targeting signal.

Some specific ubiquitin-conjugating systems exist for certain proteins whose regulated destruction is crucial for cell growth, such as cyclins and other cell-cycle control proteins. Defects in ubiquitin-dependent proteolysis have been shown to result in a variety of human diseases, including cancer, neurodegenerative diseases, and metabolic disorders. Proteasome inhibitors are showing promise as treatments for certain cancers.

Proteins are targeted to the ubiquitin/proteasome system by a number of signals. The first to be discovered is remarkably simple, consisting only of the first amino acid in the polypeptide chain. Methionine, serine, threonine, alanine, valine, cysteine, glycine and proline are protective in bacteria; the remaining 12 signal proteolytic attack. Proteins are, however, more commonly targeted to the degradative pathway by a number of other more complex internal signals (for example, phosphorylation of specific residues, denaturation or oxidative damage). For example, the transcription factor NFκB is inhibited and retained in the cytoplasm by another protein IκB, which has a short peptide motif containing two serines. Phosphorylation of these serines, which is the culmination of a complex signal transduction pathway, leads to recognition by a ubiquitin ligase, the ubiquitination of IκB and its degradation, releasing NFκB. Another example of a regulated signal for ubiquitination is seen in the transcription factor hypoxia-inducible factor, HIF-1α, which is active when oxygen levels fall below about 5% O_2. In normal oxygen conditions HIF-1α is rapidly degraded because an oxygen-sensing prolyl hydroxylase modifies it and this modification is recognized by a specific ubiquitin ligase.

Unfolded and damaged proteins are preferential substrates for ubiquitination and degradation, even where the amino-terminal amino acid is protective. Some mechanisms for regulating protein degradation include modification of the protein's amino terminus. All proteins are initially synthesized with methionine as their amino-terminal amino acid. Methionine aminopeptidases and associated enzymes remove the methionine and trim the end of the polypeptide chain until certain residues are reached. However, methionine aminopeptidases will not remove this initiator methionine if it would expose an amino acid that signals destruction.

A class of proteins exists in all cells in all organisms that bind to selected proteins to help solubilize and refold aggregates of misfolded and unfolded forms before they can be degraded. These are known as **stress-response proteins** and their synthesis is upregulated in response to heat shock and other stresses that increase the amount of protein unfolding, misfolding and aggregation in the cell.

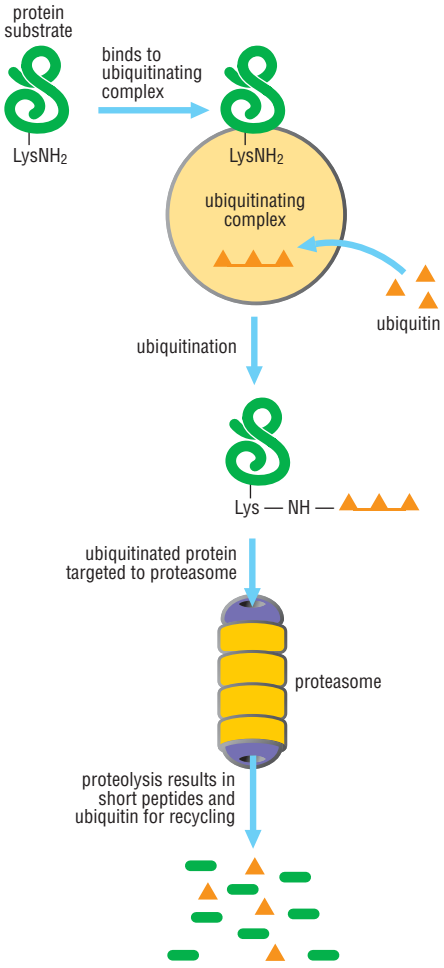

Figure 3-20 Pathway for degradation of ubiquitinated proteins A substrate protein with an exposed lysine side chain near the amino terminus is targeted by binding of a multienzyme ubiquitinating complex which, in this example, recognizes the amino-terminal amino acid of the substrate. The complex attaches polyubiquitin chains to the substrate in an ATP-dependent reaction. The polyubiquitinated substrate is then targeted to the proteasome, whose cap recognizes the ubiquitin tag. After the substrate is chopped up into peptide fragments (which may then be degraded further by other proteases), the ubiquitin is recycled.

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Web site:
http://www.chembio.uoguelph.ca/educmat/chm736/degradat.htm

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3-12 Control of Protein Function by Phosphorylation

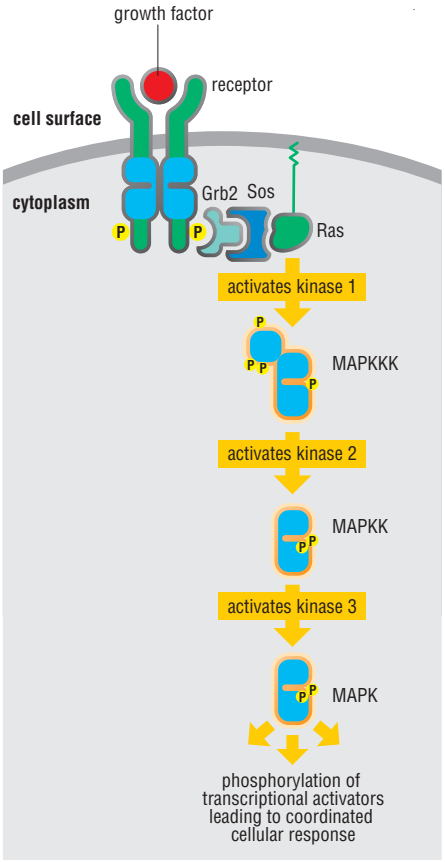

Figure 3-21 A kinase activation cascade in an intracellular signaling pathway that regulates cell growth A growth factor, or mitogen, initiates a kinase cascade by inducing dimerization of its receptor, whose cytoplasmic regions contain tyrosine kinase domains. When these domains are brought together they phosphorylate and activate one another, leading to phosphorylation of tyrosines on other parts of the receptor's cytoplasmic domains, and creating binding sites for an adaptor molecule (such as Grb2), which in turn binds a guanine-nucleotide exchange factor (Sos), bringing this into proximity with the membrane-associated G protein Ras, which is thereby activated (see Figure 3-14). Ras then activates a kinase cascade of three so-called mitogen-activated protein kinases (MAPKs): a mitogen-activated protein kinase kinase kinase (MAPKKK), which phosphorylates and activates a mitogen-activated protein kinase kinase (MAPKK), which in turn phosphorylates and activates a mitogen-activated protein kinase (MAPK). This last kinase phosphorylates a number of different gene regulatory proteins.

Protein function can be controlled by covalent modification

It is estimated that 50–90% of the proteins in the human body are post-translationally modified. Post-translational covalent modification allows the cell to expand its protein structural and functional repertoire beyond the constraints imposed by the 20 naturally encoded amino acids. More than 40 different post-translational covalent modifications have been identified in eukaryotic cells. While some are widespread, others have been observed on only a few proteins. Phosphorylation, glycosylation, lipidation, and limited proteolysis are the most common. Of the remainder, some of the most important are methylation, *N*-acetylation, attachment of the protein SUMO and nitrosylation. In the remaining sections of this chapter, we shall describe the part played by these modifications in controlling protein function.

Most covalent modifications can change the location of the protein, or its activity, or its interactions with other proteins and macromolecules. Limited proteolysis can also be deployed to amplify low-concentration or transient signals, through proteolytic cascades in which the initial stimulus activates a proteolytic enzyme which in turn activates many molecules of the next enzyme in the cascade, and so on. The complement cascade, which is activated in response to microbial cell surfaces and promotes immune defenses, is one example; the blood clotting cascade, discussed later, is another.

The commonest form of covalent modification on proteins is reversible phosphorylation of serine, threonine or tyrosine side chains and we shall discuss this first. Phosphorylation, like limited proteolysis, can produce a very large response to a relatively small signal input. A single molecule of an enzyme that is catalytically activated by phosphorylation can process many thousands of substrate molecules. If the substrate is itself another enzyme, the amplification effects can be magnified further. When phosphorylation of one enzyme causes it to become active so that it can, in turn, covalently modify another enzyme and so on, a regulatory cascade can be set up which produces a large final response very rapidly.

Unlike limited proteolysis, however, phosphorylation is reversible, and this makes it well suited as a regulatory mechanism for intracellular signaling pathways, where it is important that responses can be turned off rapidly as well as turned on (Figure 3-21). In signaling pathways, an important feature of the regulatory cascade may be the opportunity it offers for independent regulation of different downstream targets and thus for a flexible and coordinated response by the cell to the initial signal.

Phosphorylation is the most important covalent switch mechanism for the control of protein function

Post-translational modification by phosphorylation has been found in all living organisms from bacteria to humans. Target proteins are phosphorylated by the action of protein kinases and dephosphorylated by **protein phosphatases**, and this reversible modification provides a switch that controls many diverse cellular processes including metabolic pathways, signaling cascades, intracellular membrane traffic, gene transcription, and movement. The phosphoryl group derives from the terminal phosphate of nucleoside triphosphates, usually ATP. Use of separate enzymes for phosphorylation (kinases) and dephosphorylation (phosphatases) enables independent control of these events by different stimuli. Kinases that phosphorylate proteins on serine, threonine or tyrosine residues constitute the third most common domain encoded in the human genome sequence, with 575 such kinases (about 2% of the genome) identified to date. In prokaryotes, a different type of kinase domain phosphorylates proteins on histidine and aspartate

Definitions

protein phosphatase: enzyme that specifically removes phosphate groups from phosphorylated serines, threonines or tyrosines on proteins.

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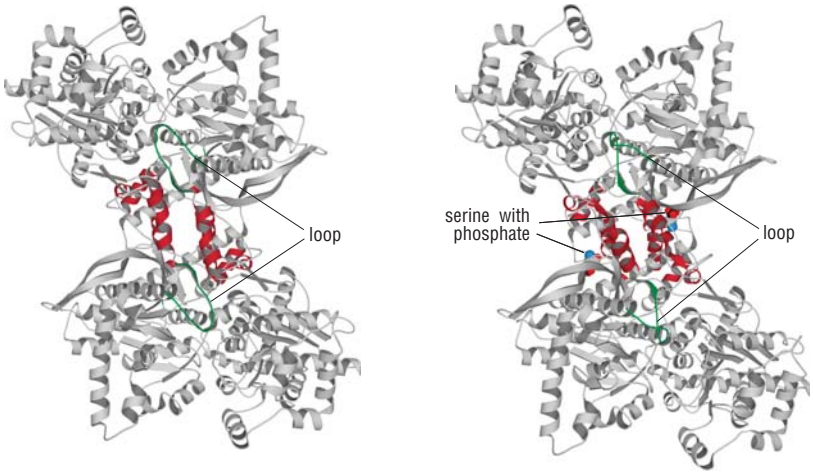


Figure 3-22 Conformational change induced by phosphorylation in glycogen phosphorylase The enzyme glycogen phosphorylase (GP) cleaves glucose units from the nonreducing ends of glycogen, the storage polymer of glucose, by phosphorolysis to produce glucose 6-phosphate. Muscle GP is a dimer of identical subunits. In response to hormonal signals the enzyme is covalently converted from the less active phosphorylase b form (left) to the more active phosphorylase a form (right) through phosphorylation of a single serine residue, serine 14, catalyzed by the enzyme phosphorylase kinase. On phosphorylation, a loop (shown in green) that sterically restricts access to the active site in the b form moves out, making the substrate-binding site more accessible. The large changes in conformation that occur at the interface between the two monomers are shown in red. (PDB 1gpa and 1gpb)

residues (although some eukaryotic-like serine/threonine and tyrosine kinases have been found in some bacteria), and the percentage of the genome devoted to regulation by phosphorylation is strikingly similar: about 1.5% of the *E. coli* genome, for example, encodes such proteins.

Covalent addition of a phosphoryl group to the side chain of serine, threonine, tyrosine, histidine or aspartic acid can have profound effects on the function of the protein. Phosphorylated residues have acquired a group that carries a double negative charge and is capable of multiple hydrogen-bonding interactions. Structural studies of phosphorylated proteins have shown that two types of interaction predominate: hydrogen bonding to main-chain amide groups at the positively polarized amino-terminal end of an alpha helix, and salt-bridging to one or more arginine residues. Other residues may also be involved in the recognition and binding of phosphoryl groups in specific cases.

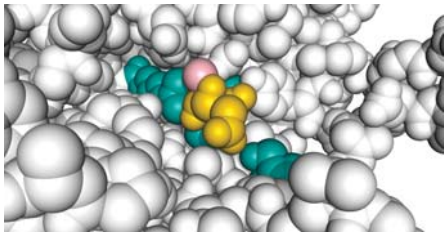
Phosphorylation can affect the target protein in two ways, which are not mutually exclusive. One effect is to change the activity of the target protein, either considerably or subtly. This change in activity may come about solely from the added bulk and charge properties of the phosphoryl group, or may result from a large conformational change in the protein, or both. The second effect of phosphorylation is to provide a new recognition site for another protein to bind (see, for example, the binding of Grb2 to the phosphorylated receptor tail in Figure 3-21). Such protein–protein interactions usually involve specialized interaction domains on the second protein that recognize the phosphorylated peptide segment; the most common domain recognizing phosphotyrosines, for example, is the SH2 domain (see section 3-1).

An instance of phosphorylation activating an enzyme by inducing a large conformational change is seen in muscle glycogen phosphorylase. Covalent attachment of a phosphoryl group to serine 14 results in a rearrangement of the amino-terminal residues in the enzyme such that the serine side chain shifts 50 Å, leading to a change in the subunit–subunit contacts of this dimeric protein, with a concomitant rearrangement of the active-site residues that activates the enzyme (Figure 3-22). The phosphoserine is stabilized in its new position primarily by salt bridges to two arginine side chains.

In contrast, the inactivation of the TCA-cycle enzyme isocitrate dehydrogenase by serine phosphorylation involves no conformational changes. Serine 113, the residue phosphorylated, is located in the active site (Figure 3-23), and attachment of a phosphoryl group inhibits binding of the negatively charged substrate isocitrate by steric exclusion and electrostatic repulsion. In isocitrate dehydrogenase the phosphoserine is stabilized by helix dipole and main-chain hydrogen-bond interactions.

One of the principal functions of reversible phosphorylation carried out by the receptor tyrosine kinases that initiate many eukaryotic signal transduction pathways is the temporary provision of new protein-interaction sites (see Figure 3-21, where phosphorylation of the receptor's own cytoplasmic domains provides a recognition site for an adaptor molecule that relays the signal onward). Another is to phosphorylate and directly activate other downstream signaling and effector molecules. The regulation of these kinases is therefore of central importance in biology, and in the next section we describe the conserved features of their activation mechanism and discuss its regulation in two important kinase families: the Src family and the cyclin-dependent kinases.

(a)



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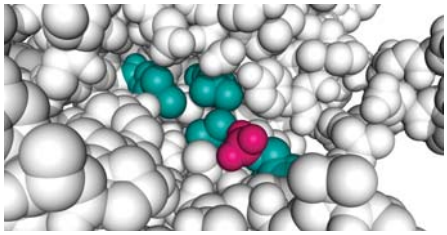

Figure 3-23 Inactivation of the active site of *E. coli* isocitrate dehydrogenase by phosphorylation The substrate-bound **(a)** and phosphorylated **(b)** states of the active site of isocitrate dehydrogenase are shown as space-filling models. The active-site residues include the serine 113 side chain, which is shown in green, the negatively charged portion of the isocitrate substrate in yellow and the phosphoryl group on serine 113 in red (in b). The phosphoryl group occupies almost the same location in the active site as the negatively charged portion of isocitrate. The phosphorylated form is inactive because isocitrate binding is both sterically blocked and electrostatically repelled by the negatively charged phosphoryl group. (PDB 5icd)

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Protein kinases are themselves controlled by phosphorylation

Phosphorylation of proteins on serine/threonine or tyrosine residues is probably the single most important regulatory mechanism in eukaryotic signal transduction, and tyrosine phosphorylation lies at the heart of control of the eukaryotic cell cycle. To a first approximation, the protein kinases responsible for phosphorylating proteins on serine, threonine and tyrosine residues all have the same fold for the catalytic domain (Figure 3-24a), although many of them also have other subunits or other domains that serve regulatory functions or target their kinase activity to specific protein substrates (Figure 3-24b). Most, but not all, of these kinases are normally inactive, and before they can phosphorylate other proteins they must themselves be activated by phosphorylation of a threonine or tyrosine residue that is located in a region termed the **activation segment**, also known as the **activation loop**. The conserved mechanism of activation of these kinases is illustrated in Figure 3-25. Kinases controlled by activation-loop phosphorylation represent some of the most important enzymes in signal transduction cascades. Two of the best understood are the Src family of tyrosine protein kinases, which are activated early in eukaryotic cell signaling pathways, and the cyclin-dependent kinases (Cdks) which coordinate the eukaryotic cell cycle. We discuss the part played by regulatory and targeting domains in the activation of Src kinases below, and in the next section we describe the activation and targeting of the cyclin-dependent kinase Cdk2.

Src kinases both activate and inhibit themselves

Src-family kinases are activated early in many signaling pathways and once activated sustain their own activated states by **autophosphorylation**, providing for a large amplification of the signal. The original Src kinase was discovered as an oncogenic variant in a tumor virus that causes sarcomas in chickens (Src is short for sarcoma). Its tumorigenic action is due to a mutation that causes unregulated autophosphorylation, leading to a sustained growth signal, and we now understand the structural basis both for the normal regulation of the kinase and for its oncogenic activation.

Src kinases recognize their target proteins through SH2 and SH3 domains that are joined to one another and to the catalytic domain by flexible linker regions (Figure 3-24b). In the absence of activating signals, these domains bind to the kinase domain, holding it in an inactive conformation (Figure 3-26): the SH2 domain binds to an inhibitory phosphate on a tyrosine

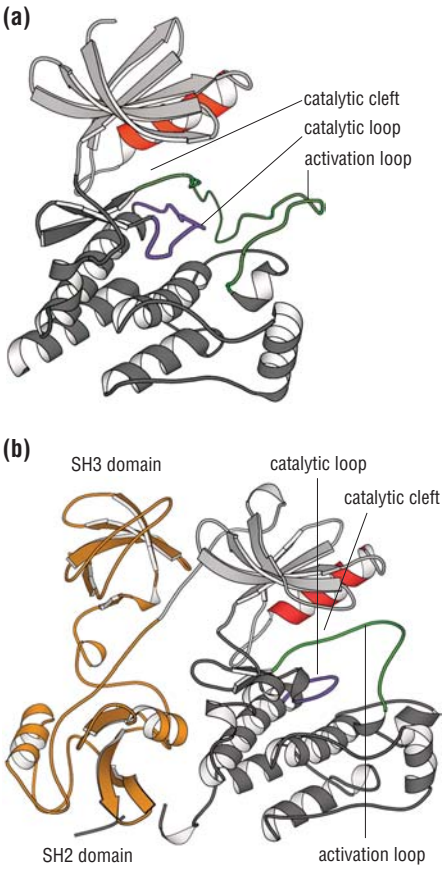

Figure 3-24 The conserved protein kinase catalytic domain (a) The catalytic domain of Lck, a signaling tyrosine kinase expressed in the cells of the immune system, is typical of the highly conserved catalytic domain of all protein kinases. This is structurally divided into two lobes, the amino-terminal (upper) lobe (light grey) being formed almost entirely from beta strands but for a single alpha helix (the C helix, red), while the carboxy-terminal (lower) lobe (dark grey) is formed almost entirely from alpha helices. Between the two lobes, which are joined by a flexible hinge, is the catalytic cleft containing the catalytic loop (purple). The activation loop, which is repositioned by phosphorylation and thereby regulates the catalytic action of the kinase, is shown in green. The flexible hinge between the two lobes is important in allowing major conformational changes that accompany activation and inhibition in some kinases. Movement of the single helical element in the amino-terminal lobe is critical to the establishment of the active conformation of the catalytic site. Kindly provided by Ming Lei and Stephen Harrison. **(b)** Structure of the Src-family tyrosine protein kinase Hck in its inactive form. The catalytic domain is attached to two small domains (gold), an SH3 domain and an SH2 domain, that target this kinase to specific protein substrates. (PDB 1ad5)

Definitions

activation loop: a stretch of polypeptide chain that changes conformation when a kinase is activated by phosphorylation and/or protein binding. This segment may or may not be the one containing the residue that is phosphorylated to activate the kinase. Usually, in the inactive state, the activation loop blocks access to the active site.

activation segment: see **activation loop**.

autophosphorylation: phosphorylation of a protein kinase by itself. Autophosphorylation may occur when the active site of the protein molecule to be phosphorylated catalyzes this reaction (*cis* autophosphorylation) or when another molecule of the same kinase provides the active site that carries out the chemistry (*trans* autophosphorylation). Autophosphorylation *in trans* often occurs when kinase molecules dimerize, a process that can be driven by ligand binding as in the receptor tyrosine kinases.

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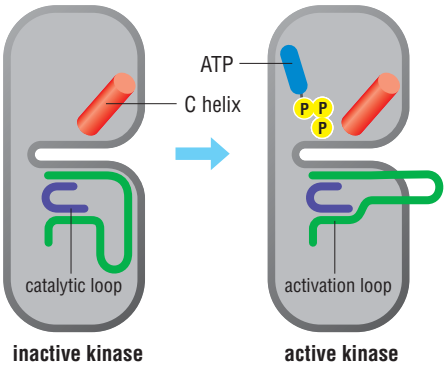

Figure 3-25 Conserved mechanism of kinase activation In all protein kinases, the activation loop (green) plays a central part in regulating catalytic activity. A conserved aspartate in the activation loop is critical to the catalytic action of the kinase, and changes in the position of the loop on phosphorylation lead to repositioning of this and other critical catalytic residues, and in some cases also regulate access of the substrate to the active-site cleft. While the active configuration of the active site (right) is essentially the same for all kinases, the inactive configuration may vary considerably. In the example schematically represented here, the activation loop swings down in the inactive conformation (left), but in some kinases it instead swings up and occludes the catalytic cleft so that substrate cannot gain access. The purple loop is the conserved catalytic loop, found in all kinases, which contains residues that participate in the chemical step of protein phosphorylation.

residue close to the carboxyl terminus of the protein, and when it is bound, the linker region joining the SH2 domain to the catalytic domain forms a polyproline helix to which the SH3 domain can bind. This clamps the catalytic domain in an inactive state from which it is released either by dephosphorylation of the tyrosine on the carboxyl tail of the protein, or by binding of the SH2 domain by a phosphotyrosine on the cytoplasmic tail of an activated receptor tyrosine kinase: this causes conformational changes that activate phosphorylation by the Src kinase by rearranging the activation loop in the active site, and at the same time releases the SH3 domain to bind to the target (Figure 3-26). The oncogenic properties of the viral Src kinase are now known to be due to a mutation of the carboxyl tail of the molecule that eliminates the tyrosine residue that is the target of the inhibitory phosphorylation.

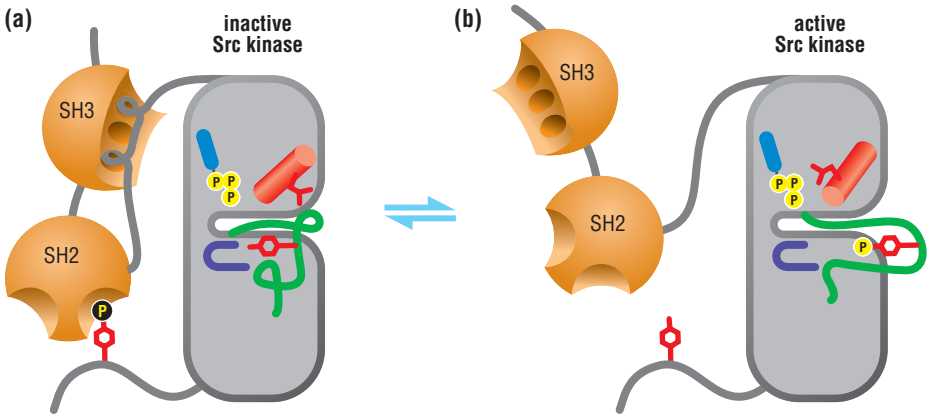

Figure 3-26 Regulation of a Src-family protein kinase In the absence of activating signals the SH2 and SH3 domains hold the kinase in an inactive conformation **(a)** in which the SH2 domain binds to an inhibitory phosphate on a tyrosine residue close to the carboxyl terminus of the protein, while the SH3 domain binds to a polyproline helix in the linker region joining the SH2 domain to the catalytic domain. **(b)** When the SH2 domain releases the carboxyl tail of the protein, either because the inhibitory phosphate is removed, or on binding a target phosphotyrosine, the polyproline helix rearranges, releasing the SH3 domain, and initiating a series of structural changes that propagate to the C helix (red) in the upper catalytic domain and the activation loop (green), which assume new conformations appropriate for substrate binding and autophosphorylation of a tyrosine in the activation loop.

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Cyclin acts as an effector ligand for cyclin-dependent kinases

Cyclin-dependent kinases (Cdks) are the enzymes that drive the cell cycle. Activated periodically during the cycle, they phosphorylate proteins that, for example, move the cell onward from growth phase to DNA replication phase or from DNA replication phase into mitosis. The activation of a Cdk is a two-step process that requires binding of a regulatory protein, the cyclin, and phosphorylation on a threonine in the activation loop of the Cdk by the enzyme Cdk-activating kinase (CAK). In eukaryotes, a Cdk known as Cdk2 regulates passage from the G1 state of the cell cycle, in which cells are growing and preparing to divide, to the S phase in which their chromosomes replicate. Cdk2 is activated by cyclin A. The cyclin subunit of the cyclin–Cdk 2 complex also targets Cdk2 to the downstream targets it phosphorylates.

In its inactive, unphosphorylated state, Cdk2 is autoinhibited by the activation loop (here called the T-loop), which partially blocks the ATP-binding site (Figure 3-27a). Structural studies of phosphorylated Cdk2 in the absence of bound cyclin, a form that has 0.3% of

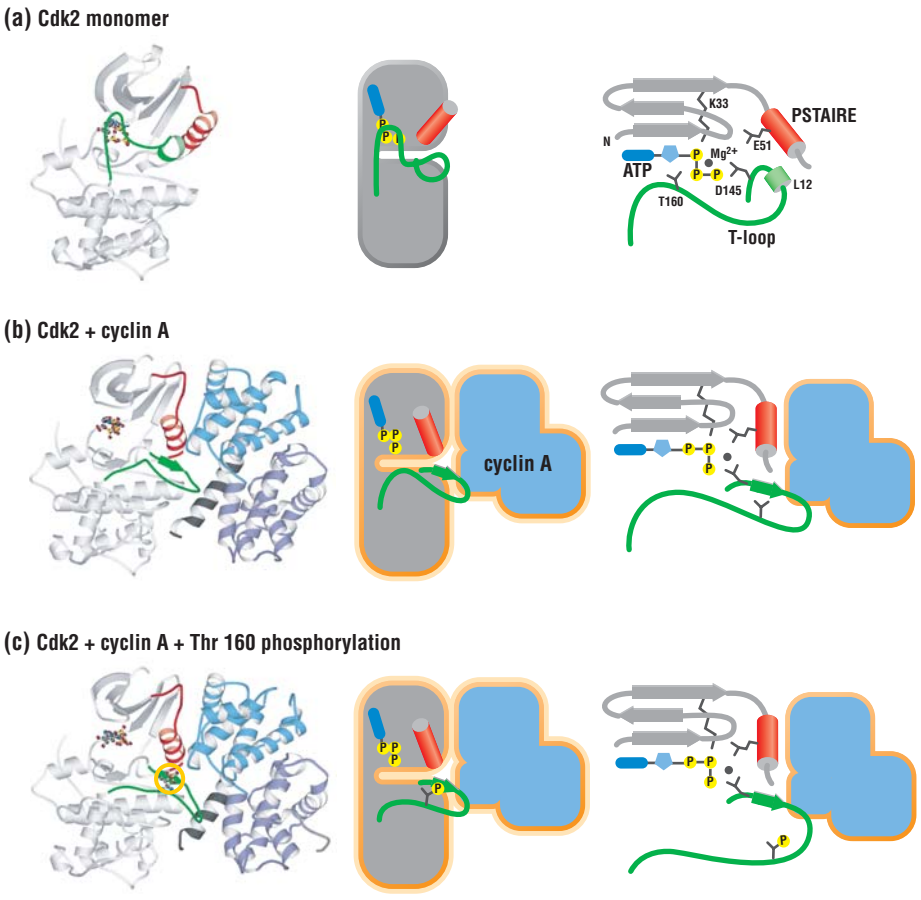

Figure 3-27 Regulation of Cdk2 activation A structure graphic representation of Cdk in various states is shown on the left, with the equivalent schematic representation in the middle and a close-up of the active site on the right. The activation loop is shown in green, with the critical helix in the upper lobe (here called PSTAIRE) in red. **(a)** In the inactive conformation of the kinase, in the absence of either cyclin or phosphorylation, a small helix (L12) in the activation loop (here called the T loop) displaces the PSTAIRE helix which contains a glutamate residue (E51) critical to catalysis. **(b)** When cyclin binds, the PSTAIRE helix moves inward and the L12 helix melts to form a beta strand, allowing E51 to interact with lysine 33 (K33) and leading to the correct orientation of the ATP phosphates. Cyclin binding also shifts the activation loop out of the active-site cleft. **(c)** Phosphorylation of threonine 160 in the activation loop flattens the activation loop, increasing its interaction with cyclin A, which allows it to interact more effectively with substrates. Taken from David O. Morgan: *The Cell Cycle: Principles of Control* (New Science Press, in the press).

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maximal enzymatic activity, show that attachment of a phosphoryl group to threonine 160 causes the activation loop to become disordered, which presumably allows ATP to bind sometimes. No other significant conformational changes are observed. The structure of the complex of unphosphorylated Cdk2 with cyclin A (Figure 3-27b), a form that is also only 0.3% active, shows some significant conformational changes, including reorganization of the activation loop. Cyclin binding and phosphorylation therefore both induce conformational changes in the protein, but these changes are different and neither alone is sufficient to fully activate the enzyme.

The structure of the phospho-Cdk2–cyclin A complex (Figure 3-27c) shows why both modifications are needed for full activity (Figure 3-28). The conformational changes seen on cyclin A binding alone all occur, but in addition the phosphothreonine side chain makes interactions that are not observed without cyclin binding. The phosphothreonine turns into the protein to interact with three arginine residues, leading to a further reorganization of the activation loop beyond that which occurs on either phosphorylation or cyclin binding alone.

This cumulative change in conformation of the activation segment is crucial for proper recognition of the substrate peptides on which this kinase acts. Specific kinases usually phosphorylate specific sequence motifs that incorporate the serine, threonine or tyrosine at which they react. Cdk2 recognizes the sequence SPXR/K. This peptide segment binds in an extended conformation across the catalytic cleft and primarily makes contact with the activation loop. In the unphosphorylated Cdk2–cyclin complex, the conformation of the activation segment does not allow the proline residue to fit properly into the active site because it would lead to a steric clash with valine 163 on the kinase. The phospho-Cdk2–cyclin A structure has a different conformation for the activation loop that repositions valine 163 out of the way. At the same time, the adjacent residue, valine 164, rotates its backbone carbonyl group away from the peptide substrate (Figure 3-28). It is this rotation that gives the kinase absolute specificity for proline in the substrate sequence motif. Proline is the only amino acid that does not have a backbone –N–H group (see Figure 1-3 in Chapter 1). Any other amino acid would have its –N–H pointing into the place where the valine 164 carbonyl group used to be, leading to an unsatisfied hydrogen bond donor in the enzyme–substrate complex, a very unfavorable situation energetically. Proline-containing substrates do not have this problem. Thus, the conformational changes in Cdk2 on phosphorylation and cyclin binding combine not only to activate the enzyme but also to allow specific recognition of the "correct" substrate.

Cdk2 provides a striking example of how protein–protein interactions combine with covalent modification by phosphorylation in regulation. This principle of multiple regulatory mechanisms operating on the same target is widely seen, especially in eukaryotic cells, and provides exquisite control of protein function.

Dephosphorylation by protein phosphatases reverses the effects of phosphorylation and it is assumed that the protein structure simply returns to its original conformation. There are many fewer phosphatases than kinases, implying that they are less specific, but some of them are known to be regulated by phosphorylation or by binding of effector molecules. Since phosphatases are in general able to recognize many phosphorylated protein substrates, it is likely that they are targeted to specific substrates as needed by mechanisms that change protein localization within the cell, as described in other sections of this chapter.

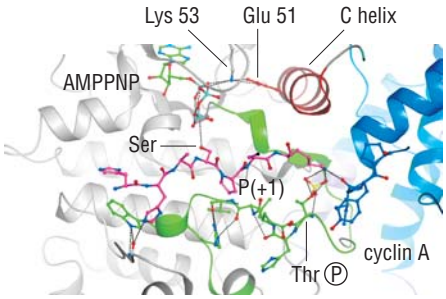

Figure 3-28 The substrate-binding site of Cdk2 The substrate to be phosphorylated (pink) binds to Cdk2 (the activation loop is shown in green) in an extended conformation. The serine to be phosphorylated (Ser) has its –OH group pointed at the gamma phosphate that will be transferred from ATP (here, the nonhydrolyzable analog AMPPNP). Activation of Cdk2 includes a change in the position of the C helix (sequence PSTAIRE, the red helix in Figure 3-27) so that glutamate 51 can interact with lysine 53 to position the ATP properly. This conformational change is induced only by the binding of cyclin A. Sequence specificity for the peptide substrate is provided by the proline residue P(+1) in the substrate. This proline comes close to the backbone of the dipeptide Val 163–Val 164 in the activation loop, which has undergone a conformational change on phosphorylation of Cdk2 to allow the substrate to fit. Graphic kindly provided by Jane Endicott and Martin Noble.

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3-15 Two-Component Signaling Systems in Bacteria

Two-component signal carriers employ a small conformational change that is driven by covalent attachment of a phosphate group

G proteins and motor ATPases are generally absent from prokaryotes, which use a different class of molecular switches. Their signaling pathways, referred to as **two-component systems**, are structured around two families of proteins. The first component is an ATP-dependent histidine protein kinase (HK). This is typically a transmembrane protein composed of a periplasmic sensor domain that detects stimuli and cytoplasmic histidine kinase domains that catalyze ATP-dependent autophosphorylation. The second component of the system, a cytoplasmic response regulator protein (RR), is activated by the histidine kinase. Signals transmitted through the membrane from the sensor domain modulate the activities of the cytoplasmic kinase domains, thus regulating the level of phosphorylation of the RR. Phosphorylation of the RR results in its activation and generation of the output response of the signaling pathway. Thus, although ATP hydrolysis is used to drive this switching process, it does so indirectly: a phosphoryl group originally derived from ATP covalently modifies the RR and serves as the trigger.

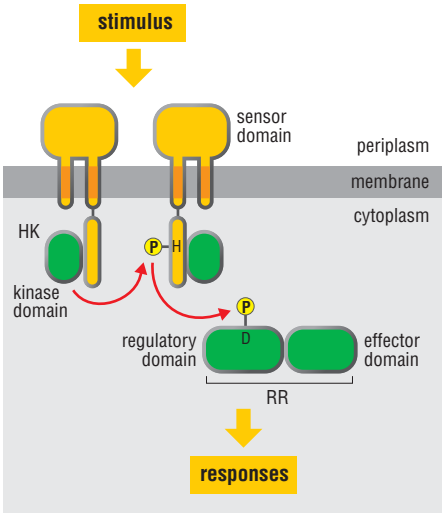

Figure 3-29 Two-component signaling mechanisms The basic two-component phosphotransfer system, found in bacteria, consists of a dimeric transmembrane receptor histidine kinase (HK) and a cytoplasmic response regulator (RR). Information flows between the two proteins in the form of a phosphoryl group (PO_3) that is transferred from the HK to the RR. HKs catalyze ATP-dependent autophosphorylation of a specific conserved His residue (H). The activities of HKs are modulated by environmental signals such as nutrients or osmotic stress. The phosphoryl group (P) is then transferred to a specific aspartic acid residue (D) located within the conserved regulatory domain of an RR. Phosphorylation of the RR typically activates an associated (or downstream) effector domain, which ultimately elicits a specific cellular response.

Two-component system proteins are abundant in most eubacterial genomes, in which they typically constitute ~1% of encoded proteins. For example, the *E. coli* genome encodes 62 two-component proteins, which are involved in regulating processes as diverse as chemotaxis, osmoregulation, metabolism and transport. In eukaryotes, two-component pathways constitute a very small number of all signaling systems. In fungi, they mediate environmental stress responses and hyphal development. In the slime mold *Dictyostelium* they are involved in osmoregulation and development, while in plants they are involved in responses to hormones and light, leading to changes in cell growth and differentiation. To date, two-component proteins have not been identified in animals and do not seem to be encoded by the human, fly or worm genomes. In most prokaryotic systems, the output response is generated directly by the RR; in many systems it functions as a transcription factor whose transcription-activating or repressing activity depends on its state of phosphorylation. Fungi and plants also contain RRs that function as transcription factors. In addition, eukaryotic two-component proteins are found at the beginning of signaling pathways where they interface with more conventional eukaryotic signaling strategies such as mitogen-activated protein (MAP) kinase and cyclic nucleotide cascades.

Similarly to most proteins in signaling pathways, two-component systems are modular in architecture. Different arrangements of conserved domains within proteins and different integration of proteins into pathways provide adaptations of the basic scheme to meet the specific regulatory needs of many different signaling systems. The prototypic prokaryotic two-component pathway (Figure 3-29) illustrates the fundamental phosphotransfer switching mechanism of both simple and more elaborate systems. Stimuli detected by the sensor domain of the histidine kinase regulate the kinase's activities. The kinase catalyzes ATP-dependent autophosphorylation of a specific histidine residue. The RR then catalyzes transfer of the phosphoryl group from this phosphorylated histidine to one of its own aspartate residues, located on the regulatory domain. Phosphorylation of the regulatory domain of the RR activates an effector domain on the same protein (or, on rare occasions, a separate effector protein) that produces the specific output response.

The regulatory domains of RRs have three activities. First, they interact with phosphorylated histidine kinase and catalyze transfer of a phosphoryl group to one of their own aspartate residues. Second, they are phosphatases that catalyze their own dephosphorylation—the counterpart to the GTPase activity of the G proteins. The phosphatase activity varies greatly among different RRs, with half-lives for the phosphorylated state ranging from seconds to hours, a span of four orders

Definitions

two-component systems: signal transduction systems found in bacteria and some eukaryotes involving a membrane-bound histidine kinase and a cytoplasmic response regulator protein that is activated by phosphorylation.

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of magnitude. And third, RRs regulate the activities of their associated effector domains (or effector proteins) in a phosphorylation-dependent manner. The different lifetimes of different regulators allow two-component signal systems to regulate a wide variety of cellular processes.

All RRs have the same general fold and share a set of conserved residues (Figure 3-30a). Phosphorylation of the active-site aspartate is associated with an altered conformation of the regulatory domain. A common mechanism appears to be involved in the structural changes that propagate from the active site. The phosphorylated aspartate is positioned by interaction of two of the phosphate oxygens with both a divalent cation (usually a magnesium ion) coordinated to three active-site carboxylate side chains and with the side chain of a conserved lysine residue. Two other highly conserved side chains have distinctly different orientations in the phosphorylated form of the regulatory domain compared to the unphosphorylated form: in the phosphorylated state a serine/threonine side chain is repositioned to form a hydrogen bond with the third phosphate oxygen and a phenylalanine/tyrosine side chain is reoriented towards the interior of the domain, filling the space that is normally occupied by the serine/threonine in the unphosphorylated protein.

Unlike the GTPases, which have large conformational changes in their switching mechanisms, the regulatory domains of two-component signaling pathways undergo less dramatic structural rearrangements on phosphorylation. Structural differences between the unphosphorylated and phosphorylated regulatory domains map to a relatively large surface involving several beta strands, alpha helices and adjacent loops, with backbone displacements ranging from 1 to 7 Å (Figure 3-30b).

In other words, like the G proteins, RR regulatory domains function as generic on–off switch modules, which can exist in two distinct structural states with phosphorylation modulating the equilibrium between them, providing a very simple and versatile mechanism for regulation. Surfaces of the regulatory domain that have altered structures in the two different conformations are exploited for protein–protein interactions that regulate effector domain function.

Not all effector domains are DNA-binding transcriptional regulators. Some function, for example, as regulators of bacterial flagellar rotation. There are many different strategies for regulation of effector domains by RR regulatory domains, including inhibition of effector domains by unphosphorylated regulatory domains, allosteric activation of effector domains by phosphorylated regulatory domains, dimerization of effector domains mediated by domain dimerization, and interaction of RRs with heterologous target effector proteins. Different RRs use different subsets of the regulatory domain surface for phosphorylation-dependent regulatory interactions. Thus, although RRs are fundamentally similar in the design of their phosphorylation-activated switch domains, there is significant versatility in the way these domains are used to regulate effector activity.

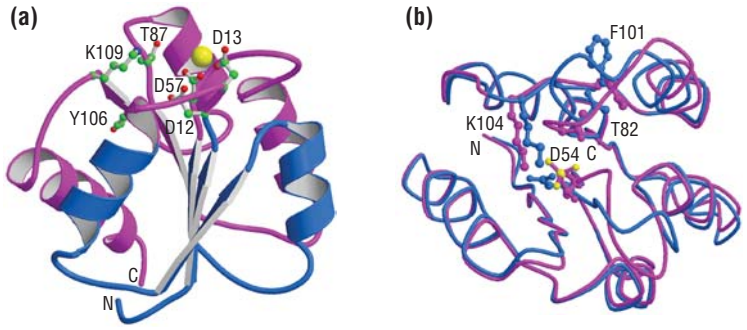

Figure 3-30 Conserved features of RR regulatory domains (a) A ribbon diagram of the bacterial RR CheY, whose protein fold is just a bare regulatory domain, is shown with ball-and-stick representations of the side chains of highly conserved residues. Residues that are highly conserved in all RR regulatory domains are clustered in two regions: an active site formed by loops that extend from the carboxy-terminal ends of strands 1, 3 and 5, and a pair of residues that form a diagonal path extending across the molecule from the active site. Three aspartic acid residues (D12, D13 and D57) position a Mg^{2+} ion (yellow) that is required for catalysis of phosphoryl transfer from an HK to aspartate 57. Three additional residues (K109, T87 and Y106) are important in propagation of the conformational change that occurs on phosphorylation. The regions of the backbone that have been observed to differ in unphosphorylated and phosphorylated regulatory domains are shown in magenta. **(b)** The conserved mechanism involved in the phosphorylation-induced conformational change is illustrated by the structures of the unphosphorylated (blue) and phosphorylated (magenta) regulatory domains of the RR protein FixJ. When D54 is phosphorylated, K104 forms an ion pair with the phosphate (yellow). T82 also forms a hydrogen bond with the phosphate and F101 has an inward orientation, positioned in the space occupied by T82 in the unphosphorylated structure. These changes trigger other rearrangements of secondary structure elements, creating a different protein-binding surface in the two states. Graphic kindly provided by Ann Stock. From West, A.H. and Stock, A.M.: *Trends Biochem. Sci.* 2001, **26**:369–376, with permission.

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Limited proteolysis can activate enzymes

Both the ubiquitin-dependent proteasome pathway discussed in section 3-11 and digestion by many other nonspecific proteases degrade polypeptide chains to small peptides or individual amino acids. But proteolysis does not have to lead to destruction and inactivation. Many proteins are post-translationally modified by limited proteolytic digestion that produces an active form from their inactive or marginally active precursors.

Limited proteolysis involves the cleavage of a target protein at no more than a few specific sites—commonly just one—usually by a specific protease. The resulting cleaved protein can have one of two fates: either the fragments remain associated, covalently (if they are disulfide-linked) or noncovalently, or they may dissociate to give two or more different polypeptides, each of which may have a completely separate fate and function. Both these outcomes are in fact observed in the maturation of the inactive precursor chymotrypsinogen to active alpha-chymotrypsin, a digestive protease (Figure 3-31). Chymotrypsinogen has the overall fold of an active serine protease with the exception that the active-site region lacks the proper configuration of main chain and catalytic side chains for both catalysis and substrate recognition. The protein is, however, "spring-loaded"; it is only the existence of a covalent bond between arginine 15 and isoleucine 16 and a set of noncovalent interactions between these residues and their neighbors that prevent it from rearranging into the correct conformation for catalysis. Specific proteolytic cleavage between arginine 15 and isoleucine 16 by the protease trypsin releases it from this constraint. After this cleavage, the amino-terminal peptide remains attached through a disulfide linkage, while the newly formed amino terminus (old isoleucine 16) swaps into a new conformation in which it makes a new set of interactions with residues of the active site, causing the active site to assume its correct catalytic configuration. The product is fully active enzymatically, although two more autocatalytic cleavages remove residues 14 and 15 and residues 147 and 148, to yield the mature form of alpha-chymotrypsin that is found in the digestive tract. The function of these latter two modifications is unknown. The activation of most serine proteases of this class from their inactive proenzyme forms occurs in a similar fashion (Figure 3-32).

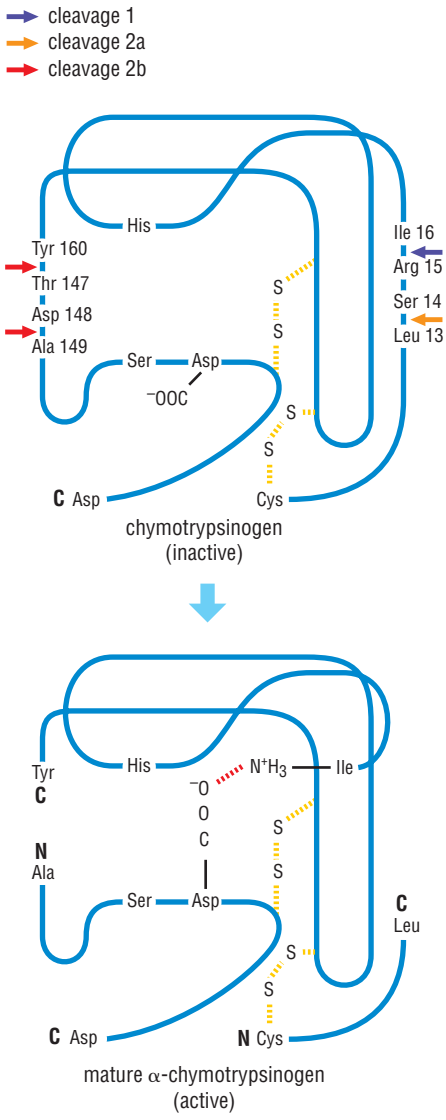

Figure 3-31 Activation of chymotrypsinogen
A schematic view of the activation of chymotrypsinogen. The polypeptide chain (blue) is held together by disulfide (–S–S–) bridges (for clarity only two of the five are shown). Cleavage between residues 15 and 16 (top) results in a rearrangement of part of the polypeptide chain (bottom) and the formation of a new interaction between isoleucine 16 and an aspartate elsewhere in the chain, which helps form the functional active site. After the first cleavage, the first 15 residues remain attached to the rest of the protein, in part by a disulfide linkage. Two more cleavage events remove residues 14–15 and residues 147–148 to produce the mature alpha-chymotrypsin.

Polypeptide hormones are produced by limited proteolysis

Limited proteolysis can also produce polypeptides with new functions, as in the production of short polypeptide hormones from long precursor proteins (Figure 3-33). All known polypeptide hormones are synthesized in "prepro" form, with a signal (pre) sequence and additional (pro) sequences that are cleaved out during maturation. Often, a single precursor sequence may contain two or more distinct hormones, each of which is released by additional cleavages. A striking example is the pituitary multihormone precursor pro-opiomelanocortin, which contains sequences for the hormones beta-lipotropin, melanocyte-stimulating hormone (MSH), endorphin, enkephalin, and adrenocorticotropic hormone (ACTH, corticotropin). ACTH is a 39-residue polypeptide that stimulates the synthesis of mineralocorticoids and glucocorticoids in the adrenal cortex, which leads to activation of steroid hormone synthesis. The other hormone products have very different target tissues and different physiological activities. Pro-opiomelanocortin is cleaved at different sites in different cell types so that they produce different spectra of hormones derived from the single precursor. Each of the cleaving enzymes is specific for a particular sequence in the precursor protein, by virtue either of its active-site structure or of the compartment in which it encounters its substrate. Hormone-processing proteases are attractive targets for drugs designed to modulate hormone-sensitive processes such as control of blood pressure and inflammation.

Definitions

proteolytic cascade: a series of protein cleavages by proteases, each cleavage activating the next protease in the cascade.

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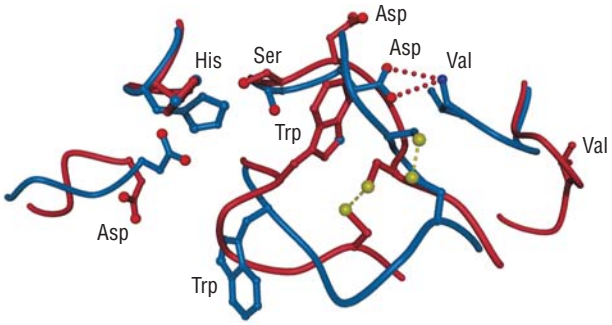

Figure 3-32 Comparison of the active sites of plasminogen and plasmin Plasminogen (red) is the inactive precursor of plasmin (blue), the primary enzyme that dissolves blood clots. Like chymotrypsinogen and many other inactive serine protease precursors, plasminogen is activated by a proteolytic cleavage. The active-site residues in plasminogen must rearrange to form an active enzyme. In chymotrypsinogen, most of the catalytic residues are already in the active configuration, but in plasminogen the entire active site is distorted. The primary cause of this distortion is a tryptophan residue that blocks the substrate-binding pocket and pushes the catalytic residues away from their correct orientations. As in all proteases of this class, the oxyanion hole involved in catalysis (see Figure 2-40) has not yet formed. Cleavage of the arginine–valine bond (not shown) in plasminogen creates a new positively charged amino-terminal amino group that interacts with the central aspartic acid residue of the oxyanion peptide, causing the peptide to rearrange into the correct configuration of the oxyanion hole. At the same time, the tryptophan swings out of the active site, unblocking the substrate-binding pocket and allowing the catalytic residues (His, Ser and Asp at the left of the diagram) to switch to the correct configuration for catalysis.

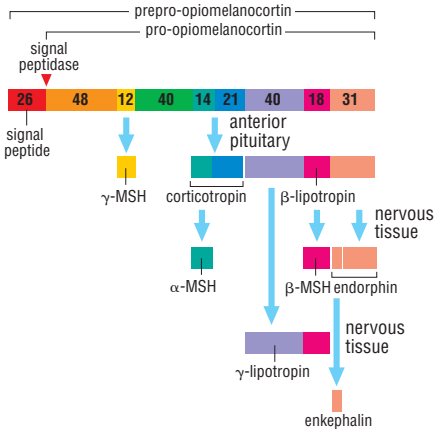

Figure 3-33 Schematic diagram of prepro-opiomelanocortin and its processing Shown are the domains that comprise the large pre-pro hormone precursor opiomelanocortin, with the number of amino acids in each domain indicated. This primary gene translation product is cleaved into many different smaller hormones by a series of proteolytic steps. First, the signal peptide that directed its secretion is cleaved off by a signal peptidase, generating pro-opiomelanocortin. Then, a number of other cleavages, which are tissue-specific, occur in different cell types to produce different ensembles of hormones. For instance, in the pituitary, cleavage occurs to generate ACTH and beta-lipotropin, while processing in the central nervous system gives endorphin and enkephalin, among other products.

Limited proteolysis can provide for enormous, extremely rapid amplification of a signal if the product of one cleavage is an active protease that can go on to activate other proteins by cleavage. Several such activations may follow one another to generate a **proteolytic cascade** in which the initial activation of a single molecule of an inactive proenzyme produces a huge final output. This is how the blood coagulation pathway works. Regardless of whether the pathway is activated internally, as in thrombosis, or at an open wound, the first step is the production of an active, specific protease from an inactive precursor. A single molecule of this activated protease is capable of activating thousands of molecules of its specific substrate, another inactive protease; each newly activated protease molecule can then activate thousands of molecules of their specific proenzyme substrate, and so on. The final product is millions of activated thrombin molecules, which cleave the soluble blood protein fibrinogen to form insoluble fibrin, which polymerizes to form the clot (Figure 3-34).

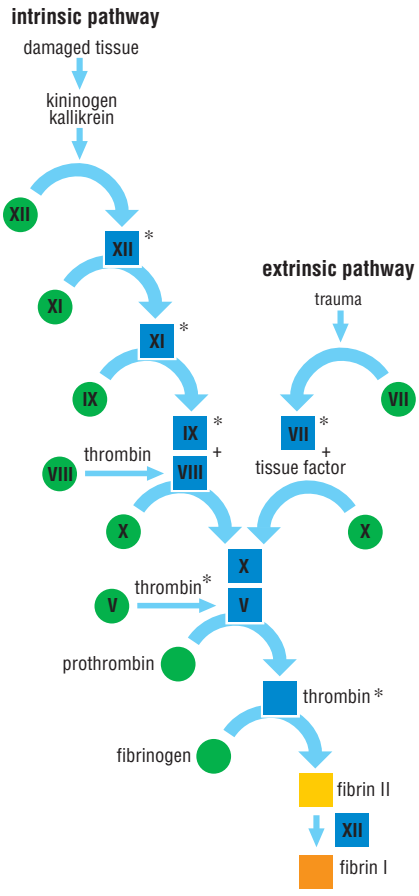

Figure 3-34 The blood coagulation cascade Each factor in the pathway is a protein that can exist in an inactive form (green circles) and an active form (blue squares), and many of these are serine proteases (denoted by an asterisk). The cascade of proteolytic activations that ends with the activation of fibrinogen to clotting fibrin is initiated by exposure of blood at damaged tissue surfaces (intrinsic pathway) or from internal trauma to blood vessels (extrinsic pathway). Although the specific initiating factors differ in the two pathways, in both cases the process begins with the conversion of an inactive serine protease to an active one. As little as one molecule of this active protease may be all that is needed to activate thousands of molecules of the next precursor protein in the cascade. Each of these can activate many more of the next, and so on down the cascade. Each protease is highly specific for a cleavage sequence in the next factor in the pathway, preventing unwanted activation. Roman numerals refer to specific proteins called factors.

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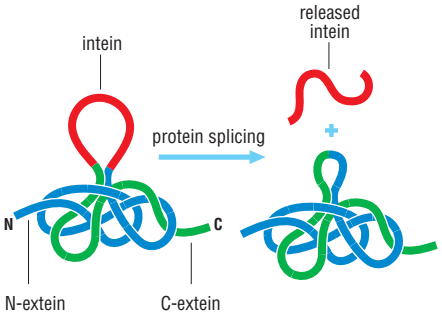


Figure 3-35 Protein splicing Schematic diagram of the process by which an internal segment of a polypeptide chain (intein) excises itself from the protein in which it is embedded, leading to the ligation of the flanking domains to yield a single polypeptide chain. Red indicates sequence to be excised (intein); blue and green are the flanking sequences. Protein splicing does not involve peptide bond breakage and religation but instead occurs entirely by peptide bond rearrangements. This allows it to proceed without a source of metabolic energy such as ATP, which would be required if the precursor protein were cleaved and religated.

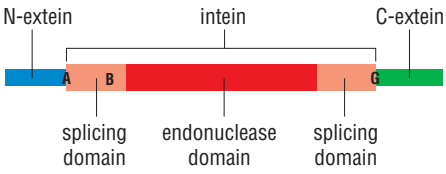
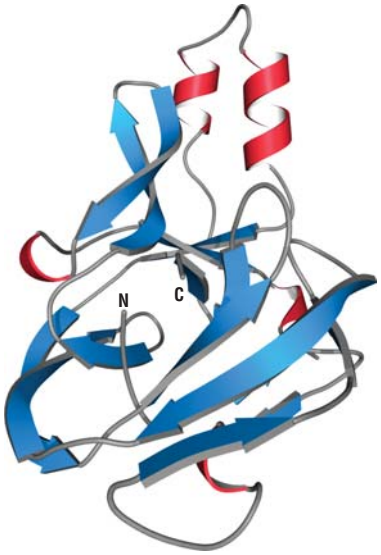


Figure 3-36 Schematic of the organization of intein-containing proteins The intein is represented by the peptide segment labeled A to G. This segment contains two splicing domains separated by a linker region that may be a homing endonuclease. Sites A, B and G represent conserved sequences important for self-splicing. Red indicates sequence to be excised (intein); blue and green are the flanking sequences.



Some proteins contain self-excising inteins

Several genes from prokaryotes and lower eukaryotes contain an in-frame open reading frame encoding an internal domain that is subsequently cleaved out from the protein to form an independent protein known as an **intein**. This process is analogous to the splicing out of introns in messenger RNA, but occurs post-translationally at the level of the protein itself. In protein splicing, internal segments in the translation product (inteins) excise themselves from the protein while ligating the flanking polypeptides (**exteins**) to form the final protein. This process generates two functional proteins from one polypeptide chain, one derived from an internal sequence in the other (Figure 3-35). The novelty of this protein splicing process is the fact that it is self-catalyzed. To date, more than 100 inteins have been discovered, approximately 70% of which reside in proteins involved in DNA replication and repair. Inteins have been found that contain as few as 134 amino acids and as many as 608. Inteins are usually composed of two distinct regions, a protein-splicing domain, which is split into two segments, and an intervening endonuclease domain (Figure 3-36). (In smaller inteins this endonuclease domain may be replaced by a short peptide linker.) The protein-splicing domain catalyzes a series of peptide bond rearrangements that lead to the excision of the entire intein from the protein as well as religation of the flanking exteins. This protein-splicing activity is autoproteolytic and requires no accessory host proteins or cofactors.

As far as is known, inteins are mobile genetic elements with no function other than their own propagation. This function depends on the endonuclease domain. It belongs to a family of enzymes called homing endonucleases. These cleave the DNA sequence corresponding to their own amino-acid sequence from the genome and then mediate its insertion into genes that lack the intein sequence. Why these elements should prefer to exist as inteins in DNA replication and repair proteins in particular is unclear.

Mutant inteins that actually cleave the protein sequence in which they occur, a reaction that does not occur in protein splicing under physiological conditions, are proving useful in protein chemistry and protein engineering. For example, tobacco plants have been genetically engineered to express a mammalian antimicrobial peptide as a fusion protein with a modified intein from vacuolar membrane ATPase. The peptide can then be purified from the plant tissue by taking advantage of the intein-mediated self-cleaving mechanism. Inteins can also be used in the semisynthesis of entire proteins for industrial and medical applications: part of the protein can be synthesized chemically, allowing for the introduction of unnatural amino acids and chemical labels, and part of it can be produced genetically. Intein sequences can then be used to couple these parts together.

The mechanism of autocatalysis is similar for inteins from unicellular organisms and metazoan Hedgehog protein

Both inteins and each of the different types of homing endonucleases are characterized by a few short signature sequence motifs and can be recognized in gene sequences. Certain amino acids are highly conserved within the splicing domain. For instance, the first amino acid on

Figure 3-37 Structure of an intein Ribbon diagram of the polypeptide chain fold of the intein from the gyrase A subunit of *Mycobacterium xenopi*. It contains an unusual beta fold in which the catalytic splice junctions are located at the ends of two adjacent antiparallel beta strands at the amino and carboxyl termini of the intein. (PDB 1am2)

Definitions

intein: a protein intron (intervening sequence). An internal portion of a protein sequence that is post-translationally excised in an autocatalytic reaction while the flanking regions are spliced together, making an additional protein product.

extein: the sequences flanking an **intein** and which are religated after intein excision to form the functional protein.

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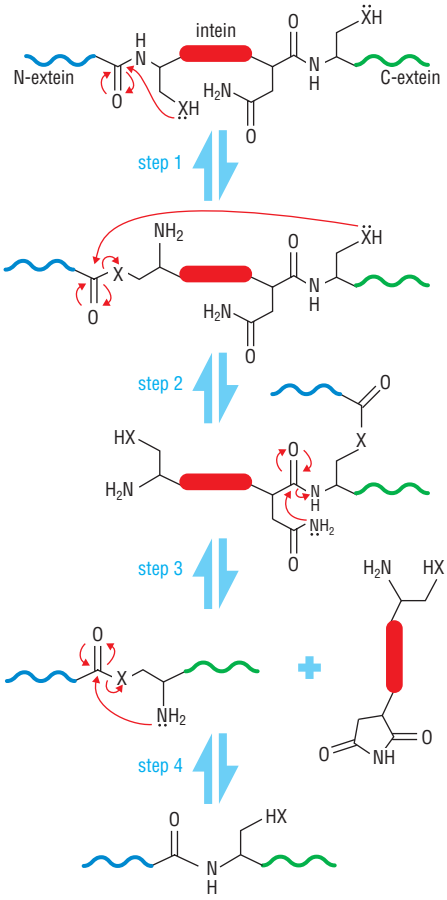

Figure 3-38 Four-step mechanism for protein splicing In step 1, a side-chain oxygen or sulfur (X) of the first intein residue (serine, cysteine or threonine; A in Figure 3-36) attacks the carbonyl group of the peptide bond that it makes with the preceding amino acid. In step 2, this carbonyl, now in the form of an ester or thioester, is attacked by the first residue of the carboxy-terminal extein segment, which is also serine, cysteine or threonine. The last residue of the intein, which is most commonly an asparagine (corresponding to position G in Figure 3-36) then cyclizes internally through its own peptide carbonyl group (step 3), thereby releasing both the intein (with a cyclic asparagine at its carboxyl terminus) and the extein, in which the amino-terminal and carboxy-terminal segments are connected via the side chain of the first residue of the carboxy-terminal segment. The spontaneous rearrangement of this ester or thioester to a normal peptide bond (step 4) completes the splicing process.

both the amino terminus of the intein domain (indicated by an A in Figure 3-36) and the carboxy-terminal extein fragment is either serine, cysteine or threonine, although an alanine has sometimes been observed. The amino acid on the carboxyl terminus of the intein (indicated by a G in Figure 3-36) is most commonly asparagine, often preceded by histidine. Finally, a ThrXXHis sequence within the amino-terminal splicing domain of the intein (indicated by a B in Figure 3-36) is usually observed. These residues have specific roles in the structure of the intein and the mechanism of protein splicing. The three-dimensional structure of an intein has now been solved for a mycobacterial protein and contains an unusual beta fold with the catalytic splice junctions at the ends of two adjacent beta strands (Figure 3-37). The arrangement of the active-site residues Ser 1 (corresponding to A in Figure 3-36), Thr 72-X-X-His 75 (corresponding to B in Figure 3-36), His 197 and Asn 198 (corresponding to G in Figure 3-36) is consistent with a proposed four-step mechanism for the protein splicing reaction (Figure 3-38).

The reaction carried out by inteins is similar to that performed by the eukaryotic Hedgehog proteins (HH), which play a central part in developmental patterning in vertebrates and invertebrates and are implicated in some important human tumors. Depending on the context, HH signals can promote cell proliferation, prevent programmed cell death, or induce particular cell fates. HH family members can exert their effects not only on cells neighboring the source of the protein signal, but also over considerable distances (up to 30 cell diameters), acting in at least some cases as classic morphogens. Such morphogens are signaling molecules that diffuse from a source to form a concentration gradient over an extended area of the target field and elicit different responses from cells according to their position within the gradient, which reflects the dosage of the ligand they are exposed to. In many cases, the precise biochemical function of the HH protein is unknown.

Like inteins, which autocatalyze their removal from inside other proteins with the concomitant joining of their flanks by a peptide bond, the carboxy-terminal domain of HH proteins autocatalyzes their cleavage from the amino-terminal domain of precursor proteins with a concomitant covalent attachment of a cholesterol molecule to the cleavage point on the amino-terminal domain. This targets the HH protein to the membrane, a process that is essential to its signaling function. The autocatalytic reactions are similar, with the cleaved peptide bond (the amino-terminal one in inteins) first changed to an ester/thioester bond that is cleaved by a nucleophilic attack from the carboxy-terminal flanking sequence in inteins or the cholesterol molecule in HH. Although these two types of self-splicing proteins are found in different types of organisms (single-celled organisms for inteins and metazoans for HH proteins), they have significant sequence similarity and their core three-dimensional structures are very similar (compare Figures 3-37 and 3-39).

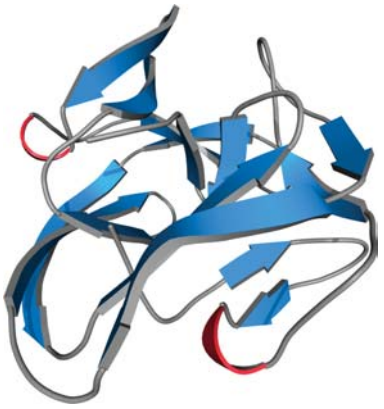

Figure 3-39 Structure of part of the Hedgehog carboxy-terminal autoprocessing domain The carboxy-terminal domain of *Drosophila* Hedgehog protein (HH-C) possesses an autoprocessing activity that results in an intramolecular cleavage of full-length Hedgehog protein and covalent attachment of a cholesterol molecule to the newly generated amino-terminal fragment. The 17 kDa fragment of HH-C (HH-C17) shown here is active in the initiation of autoprocessing. The HH-C17 structure comprises two homologous subdomains that appear to have arisen from tandem duplication of a primordial gene. Both the protein structure and the chemical features of the reaction mechanism are conserved between HH-C17 and the self-splicing regions of inteins (see Figure 3-37). (PDB 1at0)

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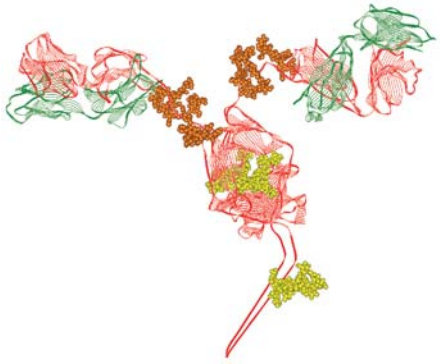

Figure 3-40 Immunoglobulin A protects mucosal surfaces from pathogenic organisms Secreted immunoglobulin A is the major antibody protecting mucosal surfaces of the gastrointestinal and respiratory tracts from pathogenic microorganisms. *N*- and *O*-linked sugars (yellow and orange respectively) protect vulnerable regions of the molecule from proteolysis. The *O*-glycosylated hinge region of IgA also provides binding sites for bacteria, protecting the mucosal surface from infection. Graphic kindly provided by Pauline Rudd. From Royle, L.M. *et al.*, unpublished.

Glycosylation can change the properties of a protein and provide recognition sites

Perhaps the most complex and diverse post-translational modifications to proteins are those that attach carbohydrate chains (oligosaccharides) to asparagine, serine and threonine residues on protein surfaces in the process known as **glycosylation**. Almost all secreted and membrane-associated proteins of eukaryotic cells are glycosylated, with oligosaccharides attached to the polypeptide chain at one or more positions by either *N*- or *O*-glycosidic bonds. There are two major types of functions for sugars attached to proteins. First, specific oligosaccharides provide recognition sites that tag glycoproteins for recognition by other proteins both inside and outside the cell and thus direct their participation in various biological processes. One important example of this is the targeting of leukocytes to sites of inflammation. Second, since oligosaccharides can be as large as the protein domains to which they are attached, they shield large areas of the protein surface, providing protection from proteases (Figure 3-40) and nonspecific protein–protein interactions. Inside the cell, oligosaccharides increase the solubility of nascent glycoproteins and prevent their aggregation.

The nature of the modification on any given protein varies from species to species and often from one tissue to the next. Glycoproteins exist as populations of glycoforms in which the same amino-acid sequence is modified by a glycosidic core with a range of different derivatives at each glycosylation site. Protein glycosylation is almost exclusively a eukaryotic property and the complexity of the modification increases as one proceeds up the evolutionary tree. Simple eukaryotes like yeast attach only a simple set of sugars; mammals modify their proteins with highly branched oligosaccharides composed of a wider range of carbohydrates. The commonest modifications are shown in Figure 3-41. The most common *N*-linked attachment consists of two *N*-acetylglucosamines with three mannoses attached; the most common *O*-linked attachment involves an *N*-acetylgalactosamine, beta-galactose core attached at serine or threonine.

For *N*-linked sugars, the core in most eukaryotes is the same, and derives from a larger preformed precursor, Glc3Man9GlcNAc2. As the protein is being synthesized in the endoplasmic reticulum (ER) the precursor is transferred to asparagine residues (N) in the glycosylation signal sequence NXS (Figure 3-42). To produce the mature oligosaccharides, the *N*-glycosylation core is first trimmed in the ER by glucosidases and mannosidases, and additional processing of the oligomannose structures can then occur in the Golgi complex (Figure 3-42), giving rise to diverse complex sugars.

In contrast to *N*-linked sugars, all of which have the same core structure, *O*-linked sugars can have at least eight different core structures, attached through *N*-acetylgalactosamine to the side chains of serine or threonine residues; these are extended by the addition of monosaccharide units.

Some viruses and bacteria use carbohydrates on cell-surface glycoproteins as Trojan horses to gain entry into the cell: they have proteins that bind specifically to these oligosaccharides, a process that leads to internalization. Antibiotics that change the structure of these carbohydrates prevent this binding, and a number of antibiotics act by blocking enzymes in glycoprotein biosynthesis, including 1-deoxymannojirimycin, which inhibits mannosidases in both the Golgi complex and the ER.

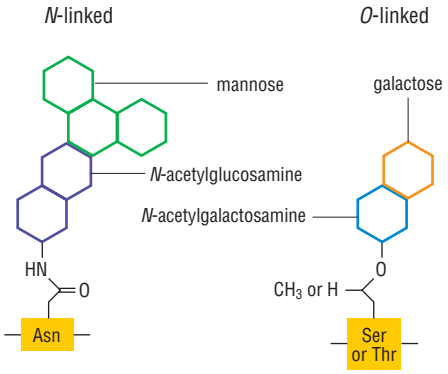

Figure 3-41 Schematic representation of the core *N*-linked oligosaccharide and a representative *O*-linked core oligosaccharide

Definitions

glycosylation: the post-translational covalent addition of sugar molecules to asparagine, serine or threonine residues on a protein molecule. Glycosylation can add a single sugar or a chain of sugars at any given site and is usually enzymatically catalyzed.

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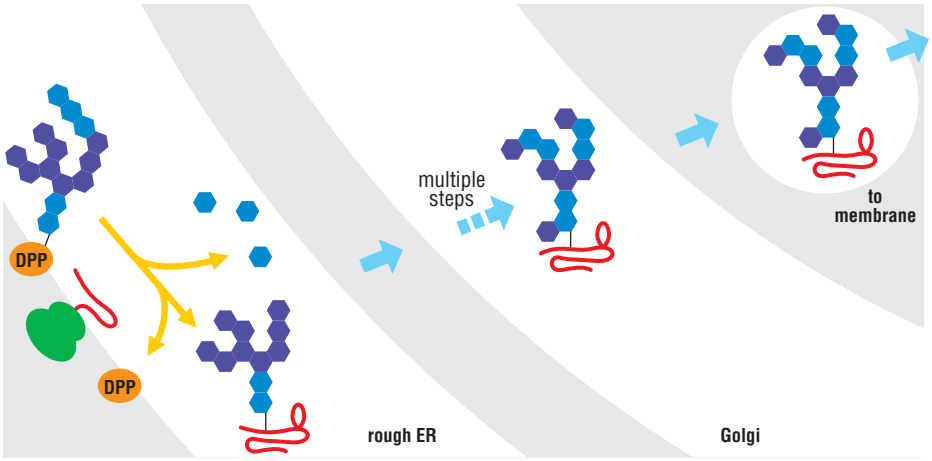

Figure 3-42 Oligosaccharide processing Schematic pathway of oligosaccharide processing on newly synthesized glycoproteins, The oligosaccharide is first transferred from its membrane-bound dolichol pyrophosphate (DPP) carrier to a polypeptide chain while the latter is still being synthesized on the ribosome. As the polypeptide chain grows within the endoplasmic reticulum (ER), monosaccharides may be cleaved from the nonreducing ends of the oligosaccharide. After completion of the synthesis of the polypeptide chain, the immature glycoprotein is carried in a transport vesicle to the Golgi apparatus where further modification occurs. New monosaccharides may be added and others removed in a multi-step process involving many different enzymes. The completed glycoprotein is finally transported to its ultimate destination.

The importance of the proper glycosylation of glycoproteins can be seen in mice lacking *N*-acetylglucosaminyltransferase I (the initial enzyme in the processing of hybrid or complex *N*-linked sugars), which die at mid-gestation, and in patients with congenital deficiencies in glycosylation, who have many pathologies ranging from neurological disorders to motor and ocular problems. Oligosaccharides attached to proteins have many roles. They may modify the activity of an enzyme, mediate the proper folding and assembly of a protein complex or facilitate the correct localization of glycoproteins; and they can be immunogenic. Glycosylation serves as an identity badge for proteins, especially those on the cell surface, indicating what type of cell or organism they come from, distinguishing mammals from their pathogens. So characteristic is this badge that many viruses bind to specific oligosaccharides as a mechanism for targeting the type of cell they infect. Because asparagine, serine and threonine-linked carbohydrates are nearly always on the outside of a folded protein they contribute to protein–protein recognition in many processes, including cell adhesion during development, immune surveillance, inflammatory reactions, and the metastasis of cancer cells. Yet there are many examples of glycoproteins that can be enzymatically stripped of their attached sugars with no effect on their biochemical function *in vitro*; in other cases, mutational loss of the oligosaccharide attachment sites has no apparent cellular consequence, and many eukaryotic proteins can be expressed in bacteria, which perform no glycosylation, and yet retain full activity *in vitro*. While this may suggest that not all glycosylations are important, it should be borne in mind that a protein enters into many interactions during its lifetime and usually only one functional assay is used to determine whether or not the sugars are important. One physical property that is dramatically affected by glycosylation is solubility: many glycoproteins become very insoluble when their sugar residues are cleaved off.

The structures of some of the most important oligosaccharide complexes have been determined by X-ray diffraction or, more commonly, by NMR. For instance, the structure of Glc3Man9GlcNac2, the precursor oligosaccharide in protein glycosylation, is shown in Figure 3-43. No obvious rules for the structures of these attached carbohydrates have yet been deduced, and in many glycoproteins whose structures have been determined, the oligosaccharides are disordered.

Another, simpler modification deserves special mention because its function has recently been elucidated. This modification is the direct enzymatic attachment of a single *N*-acetyl-glucosamine residue to a serine or threonine residue after the protein has already folded. This glycosylation is reversible by enzymatic cleavage. The sites of monoglycosylation are often identical to the sites of phosphorylation, but the effect on activity of the modified protein is usually completely different. Thus, monoglycosylation appears to transiently block phosphorylation, and is likely to be important in regulating that process. It is important to recognize that mutations that abolish phosphorylation sites may often also remove a site of monoglycosylation, and if the effects of these modifications are different then the observed phenotype will result from blocking both of them. In many breast cancer cells, monoglycosylation levels are significantly reduced owing to an increase in the synthesis of the cleaving enzyme. Less monoglycosylation would lead to more protein phosphorylation, which is also observed in most tumors and is correlated with uncontrolled growth.

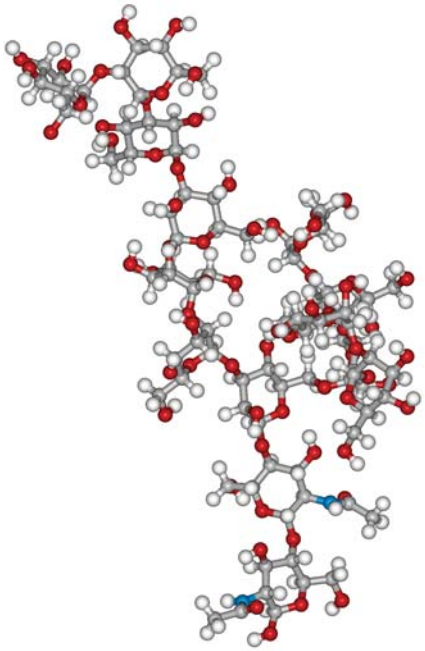

Figure 3-43 The structure of Glc3Man9GlcNac2 Average solution structure determined by NMR of the oligosaccharide involved in the early stage of glycoprotein biosynthesis. This is the 14-mer first transferred from dolichol pyrophosphate to the nascent polypeptide chain. Graphic kindly provided by Mark R. Wormald.

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3-19 Protein Targeting by Lipid Modifications

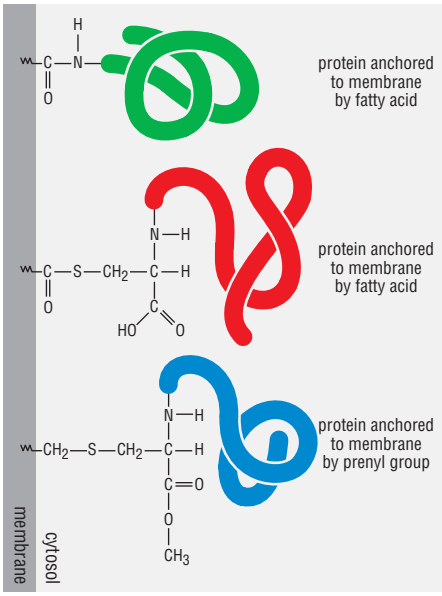

Figure 3-44 Membrane targeting by lipidation Covalent attachment of a lipid molecule to either the amino-terminal or carboxy-terminal end of a protein provides a hydrophobic anchor that localizes the protein to a membrane. The linkage is an amide (top), a thioester (middle) or a thioether (bottom) bond.

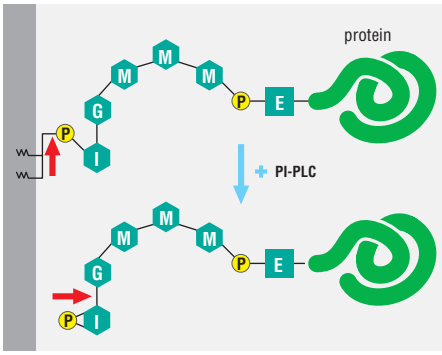

Figure 3-45 Glycosylphosphatidylinositol anchoring Schematic diagram of the reversible modification of a protein by a GPI anchor. The anchor consists of an oligosaccharide chain. The protein is connected through an amide linkage to a phosphoethanolamine molecule (E-P) that is attached to a core tetrasaccharide composed of three mannose sugars (M) and a single glucosamine sugar (G). The tetrasaccharide is in turn attached to phosphatidylinositol (I). It is the fatty-acid residues of this phosphatidylinositol group that anchor the protein to the membrane. These fatty acids may vary somewhat with the protein being modified, providing membrane specificity.

Covalent attachment of lipids targets proteins to membranes and other proteins

In signal transduction pathways and the control of protein traffic through intracellular membranes there are often many variants of one type of protein, each with a similar biochemical function. For example, more than 50 mammalian Rab proteins are known, and each of these small GTPases plays a part in directing the vesicular traffic that transports proteins between membrane-bounded compartments of the cell. To prevent chaotic cross-talk from their similar activities, each of these proteins is targeted to specific intracellular membranes at specific times in the cell cycle and during processes such as metabolism and secretion, as the need for switching functions in different compartments of the cell changes. Membrane targeting is mediated by the covalent attachment of a specific lipid group to an end of the polypeptide chain.

Lipid attachment is one of the most common post-translational modifications in eukaryotic cells. The process is sequence-specific, always involves residues either at or near either the carboxyl terminus or the amino terminus of the protein, and may require several enzymatic steps. There are four broad types of lipid modifications with distinct functional properties, classified according to the identity of the attached lipid.

These are: **myristoylation**, in which a 14-carbon fatty-acid chain is attached via a stable amide linkage to an amino-terminal glycine residue (Figure 3-44, top); **palmitoylation**, in which a 16-carbon fatty-acid chain is attached via a labile thioester linkage to cysteine residues (other fatty-acid chains can sometimes substitute for the palmitoyl group, so this modification is often called ***S*-acylation**) (Figure 3-44, middle); **prenylation**, in which a prenyl group (either a farnesyl or geranylgeranyl group) is attached via a thioether linkage to a cysteine residue initially four positions from the carboxyl terminus that becomes carboxy-terminal after proteolytic trimming and methylation of the new carboxyl terminus (Figure 3-44, bottom); and modification by a **glycosylphosphatidylinositol (GPI) anchor**, which is attached through a carbohydrate moiety (Figure 3-45).

N-myristoylated proteins include select alpha subunits of heterotrimeric G proteins, a number of non-receptor tyrosine kinases, and a few monomeric G proteins, among others. The myristoyl moiety is attached to the protein co-translationally. *S*-acylated proteins include most alpha subunits of heterotrimeric G proteins, members of the Ras superfamily of monomeric G proteins, and a number of G-protein-coupled receptors, among others. *S*-acylation is post-translational and reversible, a property that allows the cell to control the modification state, and hence localization and biological activity, of the lipidated protein. Lymphoma proprotein convertase, an enzyme that activates proproteins in the secretory pathway, occurs in both the palmitoylated and unmodified form. The palmitoylated form is degraded much faster than the unmodified one, allowing the lifetime of this enzyme to be regulated by **lipidation**. There is some overlap between *N*-myristoylated and *S*-acylated proteins such that many contain both lipid modifications. Such dual modification can have important consequences, most notably targeting of the dually modified species to distinct membrane subdomains termed lipid rafts or to caveolae; this provides a way of sublocalizing proteins to microdomains that also contain specific protein–protein interaction partners.

S-prenylation occurs in two classes, those with a single prenyl group on a cysteine residue at or near the carboxyl terminus, and those that are modified on two cysteine residues at or near the carboxyl terminus. In both cases, attachment is stable and post-translational. The difference between the two types of attachment is based on the recognition motifs recognized by the

Definitions

glycosylphosphatidylinositol anchor: a complex structure involving both lipids and carbohydrate molecules that is reversibly attached to some proteins to target them to the cell membrane.

lipidation: covalent attachment of a fatty-acid group to a protein.

myristoylation: irreversible attachment of a myristoyl group to a protein via an amide linkage.

palmitoylation: reversible attachment of a palmitoyl group to a protein via a thioester linkage.

prenylation: irreversible attachment of either a farnesyl or geranylgeranyl group to a protein via a thioether linkage.

***S*-acylation:** reversible attachment of a fatty-acid group to a protein via a thioester linkage; **palmitoylation** is an example of *S*-acylation.

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prenylating enzyme. One important protein whose activities are controlled by prenylation is the small GTPase Ras (see section 3-7). A number of *S*-prenylated proteins are also subject to *S*-acylation at a nearby cysteine residue. This type of dual modification does not apparently target the protein to the same type of membrane subdomain as dual acylation does.

A number of mammalian proteins are modified by attachment of GPI to the amino terminus. This anchor is an alternative to the lipid tails described above. GPI-anchored proteins participate in such processes as nutrient uptake, cell adhesion and membrane signaling events. All GPI-anchored proteins are destined for the cell surface via transport through the secretory pathway, where they acquire the preassembled GPI moiety. GPI modification is reversible, as the anchored protein can be released from the membrane by the action of phospholipases. In some cases, this enzyme-catalyzed release can activate an enzyme. A number of human parasites have GPI-anchored enzymes on their cell surfaces that are inactive in this state but become activated when the parasite encounters host phospholipases. By this mechanism the parasite can sense and respond to the host environment.

The enzymes involved in lipid attachment and hydrolysis are potential targets for anti-cancer, anti-fungal and other types of drugs. Several genetic diseases have been identified that involve protein lipidation. Mutations in genes encoding proteins essential for the geranyl-geranyl modification of Rab proteins are responsible for the diseases choroideremia (an incurable X-linked progressive retinal degeneration leading to blindness) and Hermansky-Pudlak syndrome (a rare disorder characterized by oculocutaneous albinism, a bleeding tendency and eventual death from pulmonary fibrosis). These diseases result from a failure to localize Rab properly.

The GTPases that direct intracellular membrane traffic are reversibly associated with internal membranes of the cell

We have already mentioned the Rab GTPases that direct transport vesicles between the membrane-bounded compartments of the cell. Two other small GTPases—Ser1 and ADP ribosylation factor (ARF)—also play an essential part in the control of vesicular traffic by recruiting the specialized coat proteins that are required for vesicle budding from the donor membrane. These small GTPases associate reversibly with their target membranes through the exposure of a covalently attached myristoyl group which occurs on exchange of GDP for GTP. We can illustrate the mechanism of vesicle formation with the ARF protein, which participates in the recruitment of vesicle coat proteins to the membrane of the Golgi complex.

ARF is myristoylated at its amino terminus and when GDP is bound this hydrophobic tail is sequestered within the protein, which therefore exists in soluble form in the cytoplasm. ARF is itself recruited to the Golgi membrane by a GTP-exchange protein, and on binding GTP, ARF undergoes a conformational change that releases the amino-terminal tail so that the protein becomes anchored to its target membrane (Figure 3-46). It is then thought to recruit preassembled coatomers—complexes of coat proteins—to the membrane, causing the coated membrane to bud off from the Golgi complex, capturing specific membrane and soluble proteins for delivery to another membrane-bounded compartment. One of the complex of coat proteins is thought to serve as a GTPase-activating protein, causing ARF to hydrolyze GTP back to GDP, withdraw its tail from the vesicle membrane, and return to the cytoplasm, with the dispersal of the coat components of the vesicle.

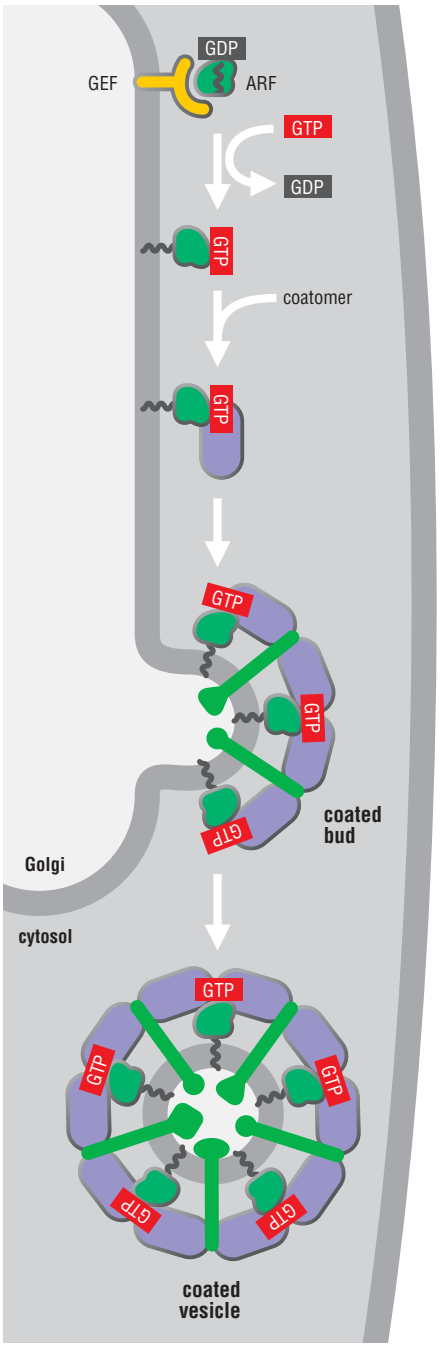

Figure 3-46 Working model for vesicular transport between Golgi compartments A key role in the process is played by the small GTPase, ARF. In its GDP-bound form, ARF is a soluble protein with a hydrophobic myristoylated tail that is tucked into the protein. When ARF is recruited to the Golgi membrane by a GTP-exchange factor, GDP is exchanged for GTP and the amino-terminal region of ARF rearranges, releasing the tail which targets ARF to the membrane. Membrane-bound ARF then recruits coat proteins necessary for vesicle budding and transport, trapping membrane and soluble proteins (not shown) in the vesicle as it pinches off.

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3-20 Methylation, *N*-acetylation, Sumoylation and Nitrosylation

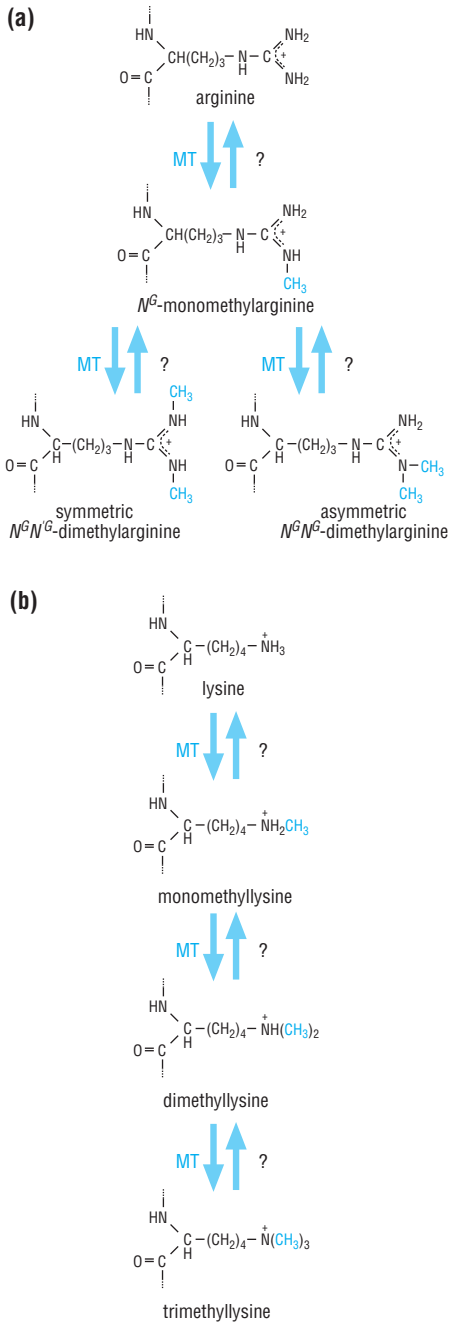

Figure 3-47 Structures of methylated arginine and lysine residues (a) Arginine may be mono- or dimethylated, either symmetrically or asymmetrically; **(b)** lysine may be mono-, di- or trimethylated. MT, methyltransferase.

Fundamental biological processes can also be regulated by other post-translational modifications of proteins

Phosphorylation, glycosylation, lipidation, and limited proteolysis are the commonest post-translational covalent modifications of proteins. However, important regulatory functions are also performed by methylation, *N*-acetylation, attachment of SUMO and nitrosylation.

Methylation occurs at arginine or lysine residues and is a particularly common modification of proteins in the nuclei of eukaryotic cells. Methylation of eukaryotic proteins is performed by a variety of methyltransferases, which use *S*-adenosylmethionine as the methyl donor. Three main forms of methylarginine have been found in eukaryotes: N^G-monomethylarginine, N^G,N^G-asymmetric dimethylarginine, and N^G,N'^G-symmetric dimethylarginine (Figure 3-47a); lysine may be mono-, di-, or trimethylated (Figure 3-47b). In contrast to phosphorylation, methylation appears to be irreversible: methylated lysine and arginine groups are chemically stable, and no demethylases have been found in eukaryotic cells. Thus regulation of this modification must occur through regulation of methyltransferase activity, and removal of the methylated proteins themselves. Arginine methylation most commonly occurs at an RGG sequence, and less often at other sites such as RXR and GRG. Although methylation does not change the overall charge on an arginine residue, it greatly alters the steric interactions this group can make and eliminates possible hydrogen-bond donors. It is not surprising, therefore, that methylation has been shown to alter protein–protein interactions. For example, asymmetric methylation of Sam68, a component of signaling pathways that recognizes proline-rich domains, decreases its binding to SH3-domain-containing but not to WW-domain-containing proteins. Arginine methylation is particularly prevalent in heterogeneous nuclear ribonucleoproteins (hnRNPs), which have roles in pre-mRNA processing and nucleocytoplasmic RNA transport. A second centrally important group of nuclear proteins to undergo methylation are the histones that package chromosomal DNA in the DNA–protein complex known as **chromatin**. Histone methylation on lysine by histone methyltransferases changes the functional state of chromatin in the region of the modification, with important effects on gene expression and DNA replication and repair. Some of these are thought to be due to effects on the compaction of the chromatin, but it is clear that others depend on the recruitment to the DNA of "silencing" proteins that recognize specific modifications to specific lysines through chromodomains characteristic of these proteins (see Figure 3-2) and suppress gene expression. Histones can also undergo phosphorylation, ubiquitination and acetylation (see below), which also affect the functions directed by chromosomal DNA.

***N*-acetylation**, which is catalyzed by one or more sequence-specific *N*-acetyltransferases, usually modifies the amino terminus of the protein backbone with an acetyl group derived from acetyl-CoA. It has been estimated that more than one-third of all yeast proteins may be so modified. This modification has a number of roles, including blocking the action of aminopeptidases and otherwise altering the lifetime of a protein in the cell. *N*-acetylation of

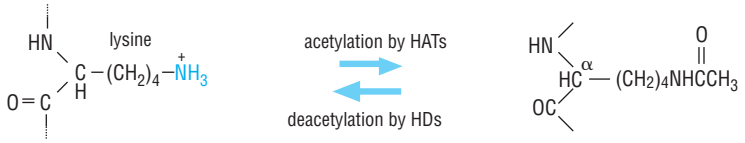

Figure 3-48 *N*-acetylation Acetylation by histone acetyltransferase (HAT) of amino-terminal lysine residues is an important regulatory modification of histone proteins. Deacetylation is catalyzed by histone deacetylases (HDs).

Definitions

chromatin: the complex of DNA and protein that comprises eukaryotic nuclear chromosomes. The DNA is wound around the outside of highly conserved histone proteins, and decorated with other DNA-binding proteins.

methylation: modification, usually of a nitrogen or oxygen atom of an amino-acid side chain, by addition of a methyl group. Some bases on DNA and RNA can also be methylated.

***N*-acetylation:** covalent addition of an acetyl group from acetyl-CoA to a nitrogen atom at either the amino terminus of a polypeptide chain or in a lysine side chain. The reaction is catalyzed by *N*-acetyltransferase.

nitrosylation: modification of the –SH group of a cysteine residue by addition of nitric oxide (NO) produced by nitric oxide synthase.

sumoylation: modification of the side chain of a lysine residue by addition of a small ubiquitin-like protein (SUMO). The covalent attachment is an amide bond between the carboxy-terminal carboxylate of SUMO and the NH_2 on the lysine side chain of the targeted protein.

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the amino terminus is usually irreversible, but the epsilon amino group on the side chain of lysine residues can also be acetylated by other, specific acetyltransferases, and this reaction can be reversed by deacetylases (Figure 3-48). In contrast to methylation, which maintains the charge on the amino group, acetylation does not. Reversible *N*-acetylation at lysine in histone proteins has a major role in the control of gene expression and other chromosomal functions: while histone methylation may induce either an active or an inactive state of chromatin, depending on the position and the nature of the methyl group, histone acetylation is always associated with an active state of chromatin: this is promoted by chromatin-remodeling enzymes recruited to the DNA by proteins containing bromodomains (see Figure 3-2) that specifically recognize acetylated lysines. Thus, in contrast to methylation, which regulates chromatin by creating non-binding surfaces for regulatory proteins on core histones, acetylation may influence genome function in part through affecting higher-order protein structure. Deregulation of chromatin modification pathways is widely observed in cancer.

Covalent attachment of one protein to another is a very common post-translational modification. In addition to ubiquitination (see section 3-11), another such modification is the attachment of the ubiquitin-like protein SUMO (small ubiquitin-related modifier), called **sumoylation** (Figure 3-49). The consensus sequence for sumoylation is ψKXE (where ψ is a hydrophobic amino acid and X is any amino acid); this is in marked contrast to ubiquitination, where no consensus sequence has ever been found. In yeast, the gene coding for the sole SUMO-like protein, Smt3, is essential for progression through the cell cycle. Septin, a GTP-binding protein that is essential for cell separation, is sumoylated during the G2/M phase of the cell cycle. Like ubiquitin, SUMO is attached to the amino group of lysine residues by specific SUMO-activating and -conjugating enzymes. Attachment of SUMO to proteins has been shown to change their subcellular localization, transcriptional activity and stability: for example, the SUMO conjugate of the RanGAP1 protein binds preferentially to the nuclear pore complex and so sumoylation appears to localize this protein. Other functions of SUMO attachment are uncertain at present.

Nitrosylation is one of only two post-translational modifications conserved throughout evolution—phosphorylation being the other. Yet it has been much less studied, in part because it has been difficult to understand how specificity of action is achieved for the reversible modification of proteins by NO groups. In general, NO modifies the –SH moiety of cysteine residues (Figure 3-50) and reacts with transition metals in enzyme active sites. Over 100 proteins are known to be regulated in this way. The majority of these are regulated by reversible *S*-nitrosylation of a single critical cysteine residue flanked by an acidic and a basic amino acid or by a cysteine in a hydrophobic environment. Cysteine residues are important for metal coordination, catalysis and protein structure by forming disulfide bonds. Cysteine residues can also be involved in modulation of protein activity and signaling events via other reactions of their –SH groups. These reactions can take several forms, such as redox events (chemical reduction or oxidation), chelation of transition metals, or *S*-nitrosylation. In several cases, these reactions can compete with one another for the same thiol group on a single cysteine residue, forming a molecular switch composed of redox, NO or metal ion modifications to control protein function. For example, the JAK/STAT signaling pathway is regulated by NO at multiple loci. NO is a diffusible gas, which modifies reactive groups in the vicinity of its production, which is catalyzed by the enzyme nitric oxide synthase. Thus, the localization of this enzyme is a key factor in which proteins will be susceptible to modification by NO. The mechanism by which this modification is reversed is unknown in many cases; some redox enzymes have been implicated; free glutathione may also be involved.

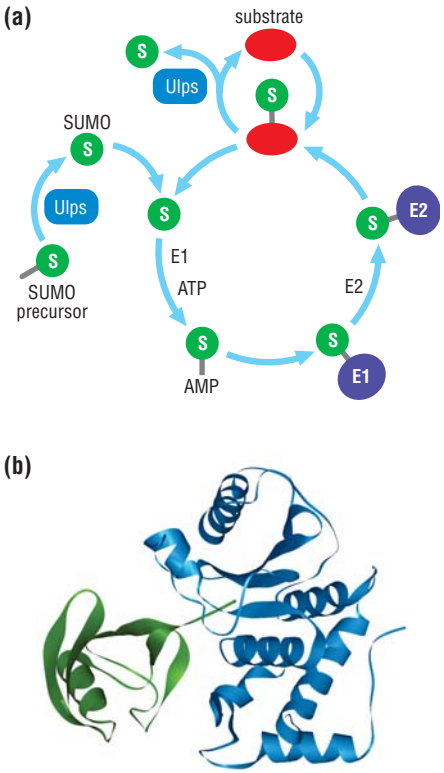

Figure 3-49 Sumoylation **(a)** In the SUMO cycle, a SUMO precursor is processed to SUMO by Ulp proteins. SUMO is then derivatized with AMP by E1, the SUMO-activating enzyme, before transfer of SUMO to a cysteine of E1 to form an E1–SUMO thioester intermediate. SUMO is passed to the SUMO-conjugating enzyme, E2, to form an E2–SUMO thioester intermediate. This latter complex is the proximal donor of SUMO to a substrate lysine in the ψKXE target sequence in the final substrate protein. SUMO can also be cleaved from sumoylated proteins by Ulp proteins. **(b)** The structure of the complex of the SUMO-binding domain of a SUMO-cleaving enzyme, Ulp1, with the yeast SUMO protein Smt3. SUMO is the small domain on the left. The size of the active-site cleft of Ulp1 allows even large SUMO–protein conjugates to bind and be cleaved.

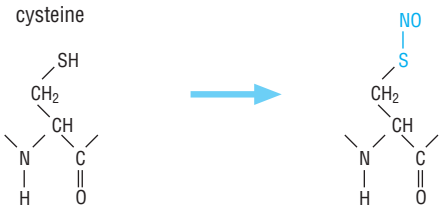

Figure 3-50 Cysteine nitrosylation

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Web resource on nitrosylation:

http://www.cell.com/cgi/content/full/106/6/675/DC1

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