CHAPTER 12 Biosignaling

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The ability of cells to receive and act on signals from beyond the plasma membrane is fundamental to life. Bacterial cells receive constant input from membrane proteins that act as information receptors, sampling the surrounding medium for pH, osmotic strength, the availability of food, oxygen, and light, and the presence of noxious chemicals, predators, or competitors for food. These signals elicit appropriate responses, such as motion toward food or away from toxic

substances or the formation of dormant spores in a nutrient-depleted medium. In multicellular organisms, cells with different functions exchange a wide variety of signals. Plant cells respond to growth hormones and to variations in sunlight. Animal cells exchange information about the concentrations of ions and glucose in extracellular fluids, the interdependent metabolic activities taking place in different tissues, and, in an embryo, the correct placement of cells during development. In all these cases, the signal represents *information* that is detected by specific receptors and converted to a cellular response, which always involves a *chemical* process. This conversion of information into a chemical change, **signal transduction**, is a universal property of living cells.

12.1 General Features of Signal Transduction

Signal transductions are remarkably specific and exquisitely sensitive. **Specificity** is achieved by precise molecular complementarity between the signal and receptor molecules **(Fig. 12-1a)**, mediated by the same kinds of weak (noncovalent) forces that mediate enzyme-substrate and antigen-antibody interactions. Multicellular organisms have an additional level of specificity, because the receptors for a given signal, or the intracellular targets of a given signal pathway, are present only in certain cell types. Thyrotropin-releasing hormone, for example, triggers responses in the cells of the anterior pituitary but not in hepatocytes, which lack receptors for this hormone. Epinephrine alters glycogen metabolism in hepatocytes but not in adipocytes; in this case, both cell types have receptors for the hormone, but whereas hepatocytes contain glycogen and the glycogen-metabolizing enzyme that is stimulated by epinephrine, adipocytes contain neither. Adipocytes respond to epinephrine by metabolizing triacylglycerols to release fatty acids, which are then transported to other tissues.

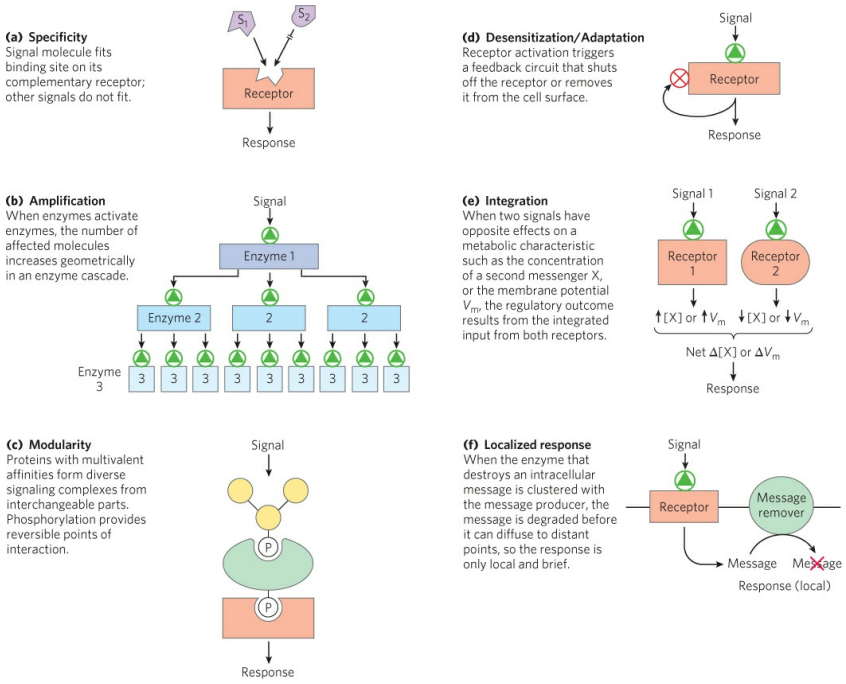


FIGURE 12-1 Six features of signal-transducing systems.

Three factors account for the extraordinary sensitivity of signal transduction: the high affinity of receptors for signal molecules, cooperativity (often but not always) in the ligand-receptor interaction, and

amplification of the signal by enzyme cascades. The **affinity** between signal (ligand) and receptor can be expressed as the dissociation constant K_d, commonly 10^{-7} M or less—meaning that the receptor detects micromolar to nanomolar concentrations of a signal molecule.

Cooperativity in receptor-ligand interactions results in large changes in receptor activation with small changes in ligand concentration (recall the effect of cooperativity on oxygen binding to hemoglobin; see Fig. 5-12). **Amplification** results when an enzyme is activated by a signal receptor and, in turn, catalyzes the activation of many molecules of a second enzyme, each of which activates many molecules of a third enzyme, and so on, in a so-called **enzyme cascade** (Fig. 12-1b). Such cascades can produce amplifications of several orders of magnitude within milliseconds. The response to a signal must also be terminated, such that the downstream effects are in proportion to the strength of the original stimulus.

Interacting signaling proteins are **modular**. Many signaling proteins have multiple domains that recognize specific features in other proteins, or in the cytoskeleton or plasma membrane. This modularity allows a cell to mix and match a set of signaling molecules to create a wide variety of multienzyme complexes with different functions or cellular locations. One common theme in these interactions is the binding of one modular signaling protein to phosphorylated residues in another protein; the resulting interaction can be regulated by phosphorylation or dephosphorylation of the protein partner (Fig. 12-1c). Nonenzymatic **scaffold proteins** with affinity for several enzymes that interact in cascades bring these enzymes together, ensuring that they interact at specific cellular locations and at specific times. Many of the domains involved in protein-protein interactions are intrinsically disordered (see Fig. 4-22), capable of folding differently depending on which protein they interact with. As a result, a single protein can have multiple functions in signaling pathways.

The sensitivity of receptor systems is subject to modification. When a signal is present continuously, the receptor system becomes **desensitized** (Fig. 12-1d), so that it no longer responds to the signal. When the stimulus falls below a certain threshold, the system again becomes sensitive. Think of what happens to your visual transduction system when you walk from bright sunlight into a darkened room or from darkness into the light.

Signal **integration** (Fig. 12-1e) is the ability of the system to receive multiple signals and produce a unified response appropriate to the combined needs of the cell or organism. Different signaling pathways

converse with each other at several levels, generating complex cross talk that maintains homeostasis in the cell and the organism.

A final noteworthy feature of signal-transducing systems is **response localization** within a cell (Fig. 12-1f). When the components of a signaling system are confined to a specific subcellular structure (a raft in the plasma membrane, for example), a cell can regulate a process locally, without affecting distant regions of the cell.

One of the revelations of research on signaling is the remarkable degree to which signaling mechanisms have been conserved during evolution. Although the number of different biological signals is probably in the thousands (Table 12-1 lists a few important types), and the kinds of response elicited by these signals are comparably numerous, the machinery for transducing all of these signals is built from about 10 basic types of protein components.

In this chapter we examine some examples of the major classes of signaling mechanisms, looking at how they are integrated in specific biological functions such as responses to hormones and growth factors; the senses of sight, smell, and taste; the transmission of nerve signals; and control of the cell cycle. Often, the end result of a signaling pathway is the phosphorylation of a few specific target-cell proteins, which changes their activities and thus the activities of the cell. Throughout our discussion we emphasize the conservation of fundamental mechanisms for the transduction of biological signals and the adaptation of these basic mechanisms to a wide range of signaling pathways.

TABLE 12-1 Some Signals to Which Cells Respond

Antigens	Light
Cell surface glycoproteins/oligosaccharides	Mechanical touch
Developmental signals	Microbial, insect pathogens
Extracellular matrix components	Neurotransmitters
	Nutrients
Growth factors	Odorants
Hormones	Pheromones
Hypoxia	Tastants

We consider the molecular details of several representative signal-transduction systems, classified according to the type of receptor. The trigger for each system is different, but the general features of signal transduction are common to all: a signal interacts with a receptor; the activated receptor interacts with cellular machinery, producing a second signal or a change in the activity of a cellular protein; the metabolic activity of the target cell undergoes a change; and finally, the transduction event ends. To illustrate these general features of signaling systems, we look at examples of four basic receptor types **(Fig. 12-2)**.

1. *G protein–coupled receptors* that *indirectly* activate (through GTP-binding proteins, or G proteins) enzymes that generate intracellular second messengers. This type of receptor is illustrated by the *β*-adrenergic receptor system that detects epinephrine (adrenaline) (Section 12.2). Vision, olfaction, and gustation are sensory systems that also operate through G protein–coupled receptors (Section 12.3).
2. *Receptor enzymes* in the plasma membrane that have an enzymatic activity on the cytoplasmic side, triggered by ligand binding on the extracellular side. Receptors with tyrosine kinase activity, for example, catalyze the phosphorylation of Tyr residues in specific intracellular target proteins. The insulin receptor is one example (Section 12.4); the receptor for epidermal growth factor (EGFR) is another. Receptor guanylyl cyclases also fall in this general class (Section 12.5).
3. *Gated ion channels* of the plasma membrane that open and close (hence the term “gated”) in response to the binding of chemical ligands or changes in transmembrane potential. These are the simplest signal transducers.
4. *Nuclear receptors* that bind specific ligands (such as the hormone estrogen) and alter the rate at which specific genes are transcribed and translated into cellular proteins. Because steroid hormones function through mechanisms intimately related to the regulation of gene expression, we consider them only briefly here (Section 12.8) and defer a detailed discussion of their action until Chapter 28.

As we begin this discussion of biological signaling, a word about the nomenclature of signaling proteins is in order. These proteins are typically discovered in one context and named accordingly, then prove to be involved in a broader range of biological functions for which the original name is not helpful. For example, the retinoblastoma protein, pRb, was initially identified as the site of a mutation that contributes to cancer of the

retina (retinoblastoma), but it is now known to function in many pathways essential to cell division in all cells, not just those of the retina. Some genes and proteins are given noncommittal names: the tumor suppressor protein p53, for example, is a *p*rotein of *53* kDa, but its name gives no clue to its great importance in the regulation of cell division and the development of cancer. In this chapter we generally define these protein names as we encounter them, introducing the names commonly used by researchers in the field. Don't be discouraged if you can't get them all straight the first time you encounter them.

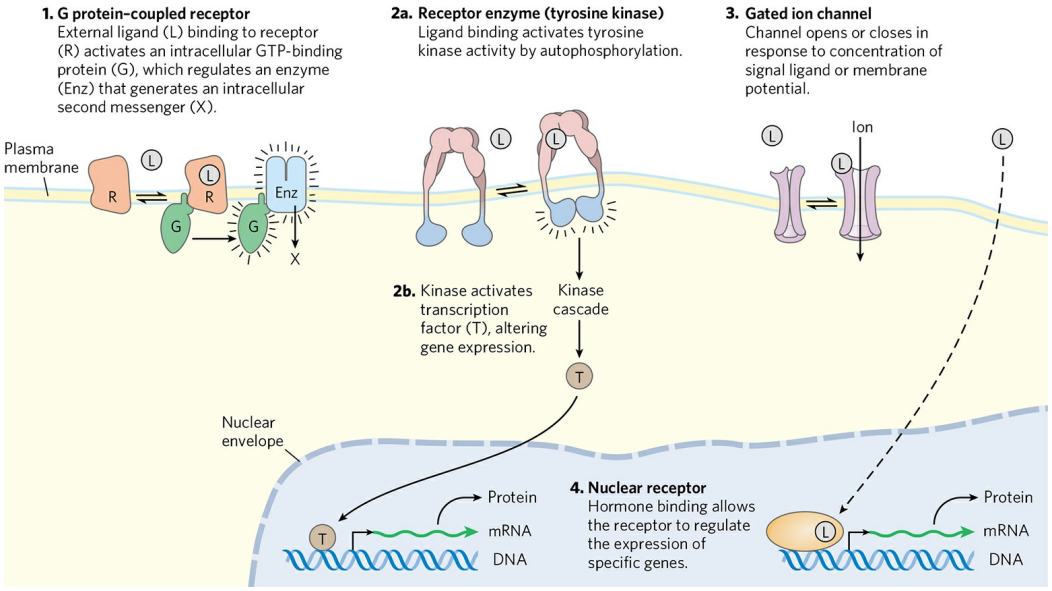


FIGURE 12-2 Four general types of signal transducers.

SUMMARY 12.1 General Features of Signal Transduction

- All cells have specific and highly sensitive signal-transducing mechanisms, which have been conserved during evolution.
- A wide variety of stimuli act through specific protein receptors in the plasma membrane.
- The receptors bind the signal molecule and initiate a process that amplifies the signal, integrates it with input from other receptors, and transmits the information throughout the cell, or in some cases to a local region of the cell. If the signal persists, receptor desensitization reduces or ends the response.
- Multicellular organisms have four general types of signaling mechanisms: plasma membrane proteins that act through G proteins, receptors with internal enzyme activity (such as tyrosine kinase), gated ion

channels, and nuclear receptors that bind steroids and alter gene expression.

12.2 G Protein–Coupled Receptors and Second Messengers

As their name implies, **G protein–coupled receptors (GPCRs)** are receptors that act through a member of the **guanosine nucleotide–binding protein**, or **G protein**, family. Three essential components define signal transduction through GPCRs: a plasma membrane receptor with seven transmembrane helical segments, a G protein that cycles between active (GTP-bound) and inactive (GDP-bound) forms, and an effector enzyme (or ion channel) in the plasma membrane that is regulated by the activated G protein. An extracellular signal such as a hormone, growth factor, or neurotransmitter is the "first messenger" that activates a receptor from outside the cell. When the receptor is activated, its associated G protein exchanges its bound GDP for a GTP from the cytosol. The G protein then dissociates from the activated receptor and binds to the nearby effector enzyme, altering its activity. The effector enzyme then causes a change in the cytosolic concentration of a low molecular weight metabolite or inorganic ion, which acts as a **second messenger** to activate or inhibit one or more downstream targets, often protein kinases.

The human genome encodes just over 800 GPCRs, about 350 for detecting hormones, growth factors, and other endogenous ligands, and perhaps 500 that serve as olfactory (smell) and gustatory (taste) receptors. GPCRs have been implicated in many common human conditions, including allergies, depression, blindness, diabetes, and various cardiovascular defects, with serious health consequences. GPCR mutations are also found in 20% of all cancers. More than a third of *all* drugs on the market target one GPCR or another. For example, the *β*-adrenergic receptor, which mediates the effects of epinephrine, is the target of the "beta blockers," prescribed for such diverse conditions as hypertension, cardiac arrhythmia, glaucoma, anxiety, and migraine headache. More than 100 of the GPCRs found in the human genome are still "orphan receptors," meaning that their natural ligands are not yet identified, and so we know nothing about their biology. The *β*-adrenergic receptor, with well-understood biology and pharmacology, is the prototype for all GPCRs, and our discussion of signal-transducing systems begins there. ■

The *β*-Adrenergic Receptor System Acts through the Second Messenger cAMP

Epinephrine sounds the alarm when a threat requires the organism to mobilize its energy-generating machinery; it signals the need to fight or flee. Epinephrine action begins when the hormone binds to a protein receptor in the plasma membrane of an epinephrine-sensitive cell. **Adrenergic receptors** ("adrenergic" reflects the alternative name for epinephrine, adrenaline) are of four general types, α_1, α_2, β_1, and β_2, defined by differences in their affinities and responses to a group of agonists and antagonists. **Agonists** are molecules (natural ligands or their structural analogs) that bind to a receptor and produce the effects of the natural ligand; **antagonists** are analogs that bind the receptor without triggering the normal effect and thereby block the effects of agonists, including the natural ligand. In some cases, the affinity of a synthetic agonist or antagonist for the receptor is greater than that of the natural agonist **(Fig. 12-3)**. The four types of adrenergic receptors are found in different target tissues and mediate different responses to epinephrine. Here we focus on the **β-adrenergic receptors** of muscle, liver, and adipose tissue. These receptors mediate changes in fuel metabolism, as described in Chapter 23, including the increased breakdown of glycogen and fat. Adrenergic receptors of the β_1 and β_2 subtypes act through the same mechanism, so in our discussion, "β-adrenergic" applies to both types.

Like all GPCRs, the β-adrenergic receptor is an integral protein with seven hydrophobic, helical regions of 20 to 28 amino acid residues that span the plasma membrane seven times, thus the alternative name for GPCRs: **heptahelical receptors**. The binding of epinephrine to a site on the receptor deep within the plasma membrane (**Fig. 12-4a**, step 1) promotes a conformational change in the receptor's intracellular domain that affects its interaction with an associated G protein, promoting the dissociation of GDP and binding of GTP from the cytosol (step 2). For all GPCRs, the G protein is heterotrimeric, composed of three different subunits: α, β, and γ. These G proteins are therefore known as **trimeric G proteins**. In this case, it is the α subunit that binds GDP or GTP and transmits the signal from the activated receptor to the effector protein. Because this G protein activates its effector, it is referred to as a **stimulatory G protein**, or $\mathbf{G_s}$. Like other G proteins (Box 12-1), G_s functions as a biological "switch": when the nucleotide-binding site of G_s (on the α subunit) is occupied by GTP, G_s is turned on and can activate its effector protein (adenylyl cyclase in the present case); with GDP bound to the site, G_s is switched off. In the active form, the β and γ subunits of G_s

dissociate from the α subunit as a $\beta\gamma$ dimer, and $G_{s\alpha}$, with its bound GTP, moves in the plane of the membrane from the receptor to a nearby molecule of adenylyl cyclase (step 3). $G_{s\alpha}$ is held to the membrane by a covalently attached palmitoyl group (see Fig. 11-13).

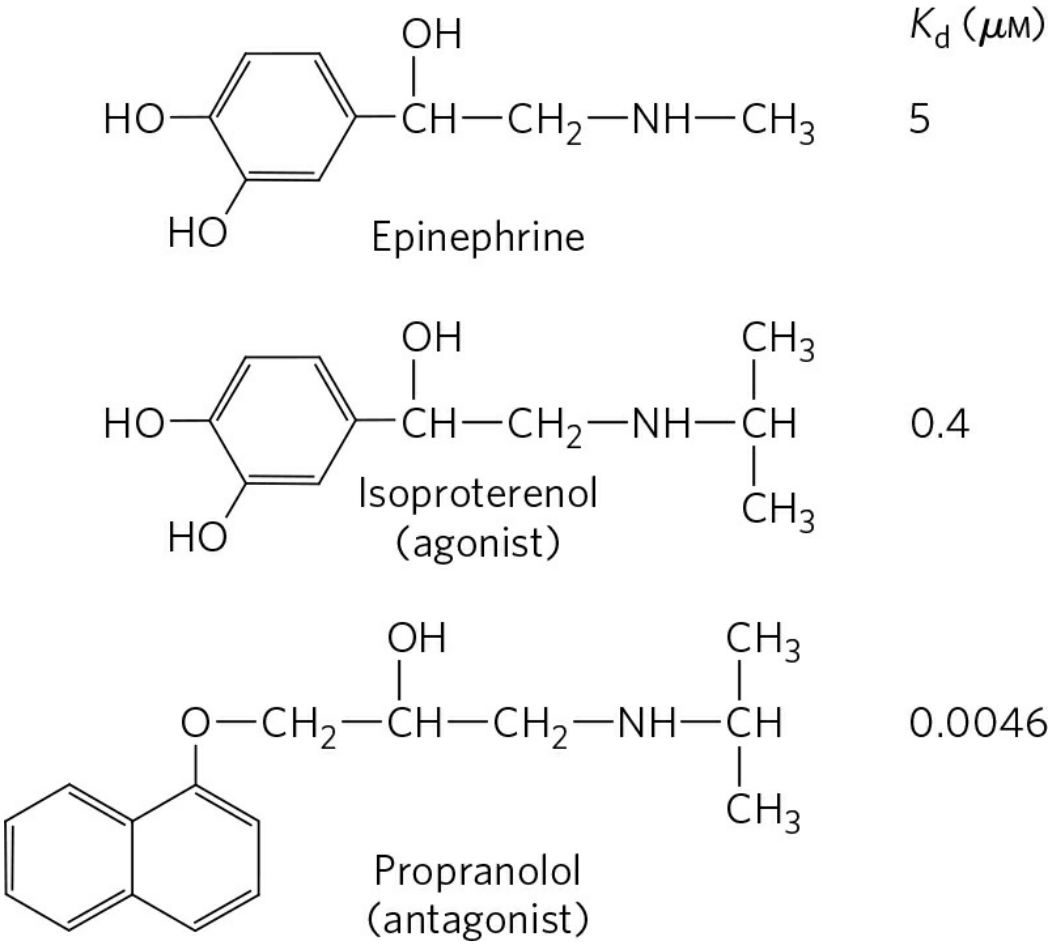


FIGURE 12-3 Epinephrine and its synthetic analogs. Epinephrine, also called adrenaline, is released from the adrenal gland and regulates energyyielding metabolism in muscle, liver, and adipose tissue. It also serves as a neurotransmitter in adrenergic neurons. Its affinity for its receptor is expressed as a dissociation constant for the receptor-ligand complex. Isoproterenol and propranolol are synthetic analogs, one an agonist with an affinity for the receptor that is higher than that of epinephrine, and the other an antagonist with extremely high affinity.

Adenylyl cyclase is an integral protein of the plasma membrane, with its active site on the cytoplasmic face. The association of active $G_{s\alpha}$ with adenylyl cyclase stimulates the cyclase to catalyze the synthesis of second messenger cAMP from ATP (Fig. 12-4a, step 4; Fig. 12-4b), raising the cytosolic [cAMP]. The interaction between $G_{s\alpha}$ and adenylyl cyclase is

possible only when $G_{s\alpha}$ is bound to GTP. The mammalian genome encodes nine isozymes of membrane-localized adenylyl cyclase, all with highly conserved sequences but, presumably, with discrete functions.

The stimulation by $G_{s\alpha}$ is self-limiting; *$G_{s\alpha}$ has intrinsic GTPase activity that inactivates $G_{s\alpha}$* by converting its bound GTP to GDP **(Fig. 12-5)**. The now inactive $G_{s\alpha}$ dissociates from adenylyl cyclase, rendering the cyclase inactive. $G_{s\alpha}$ reassociates with the $\beta\gamma$ dimer ($G_{s\beta\gamma}$), and inactive G_s is again available to interact with a hormone-bound receptor.

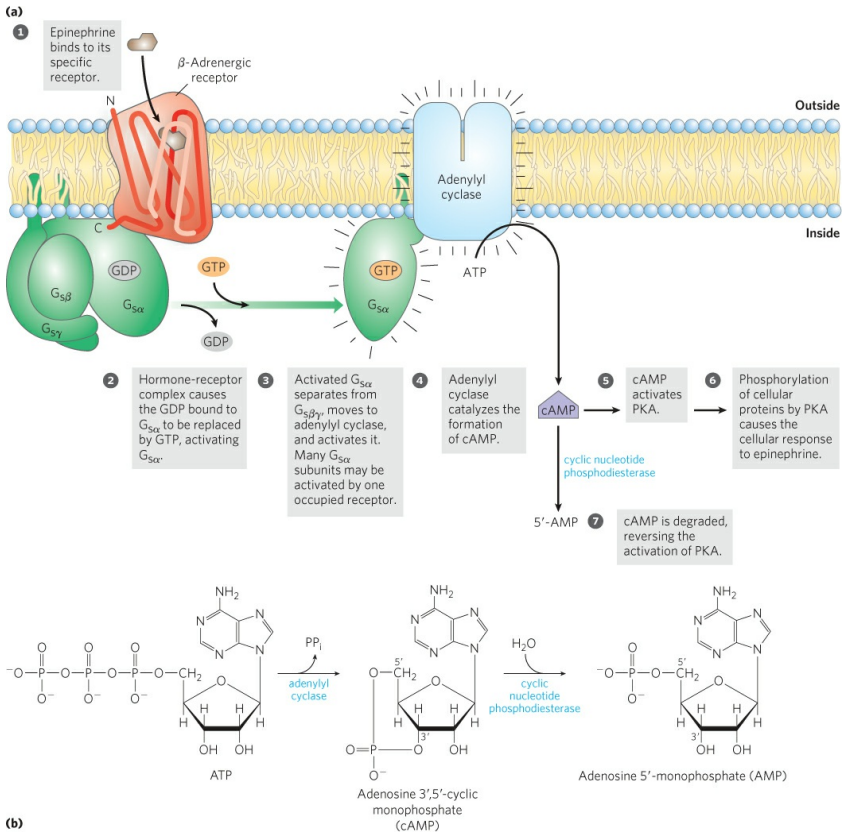


FIGURE 12-4 Transduction of the epinephrine signal: the *β*-adrenergic pathway. (a) The mechanism that couples binding of epinephrine to its receptor with activation of adenylyl cyclase; the seven steps are discussed in the text. The same adenylyl cyclase molecule in the plasma membrane may be regulated by a stimulatory G protein (G_s), as shown, or by an inhibitory G protein (G_i, not shown). G_s and G_i are under the influence of different hormones. Hormones that induce GTP binding to G_i cause *inhibition* of adenylyl cyclase, resulting in lower cellular [cAMP]. **(b)** The combined action of the enzymes that catalyze steps 4 and 7, synthesis and hydrolysis of cAMP by adenylyl cyclase and cAMP phosphodiesterase, respectively.

The role of $G_{s\alpha}$ in serving as a biological "switch" protein is not unique. A variety of G proteins act as binary switches in signaling systems with GPCRs and in many processes that involve membrane fusion or fission (Box 12-1).

Epinephrine exerts its downstream effects through the increase in [cAMP] that results from activation of adenylyl cyclase. Cyclic AMP, the second messenger, allosterically activates **cAMP-dependent protein kinase**, also called **protein kinase A** or **PKA** (Fig. 12-4a, step 5), which catalyzes the phosphorylation of specific Ser or Thr residues of targeted proteins, including glycogen phosphorylase *b* kinase. The latter enzyme is active when phosphorylated and can begin the process of mobilizing glycogen stores in muscle and liver in anticipation of the need for energy, as signaled by epinephrine.

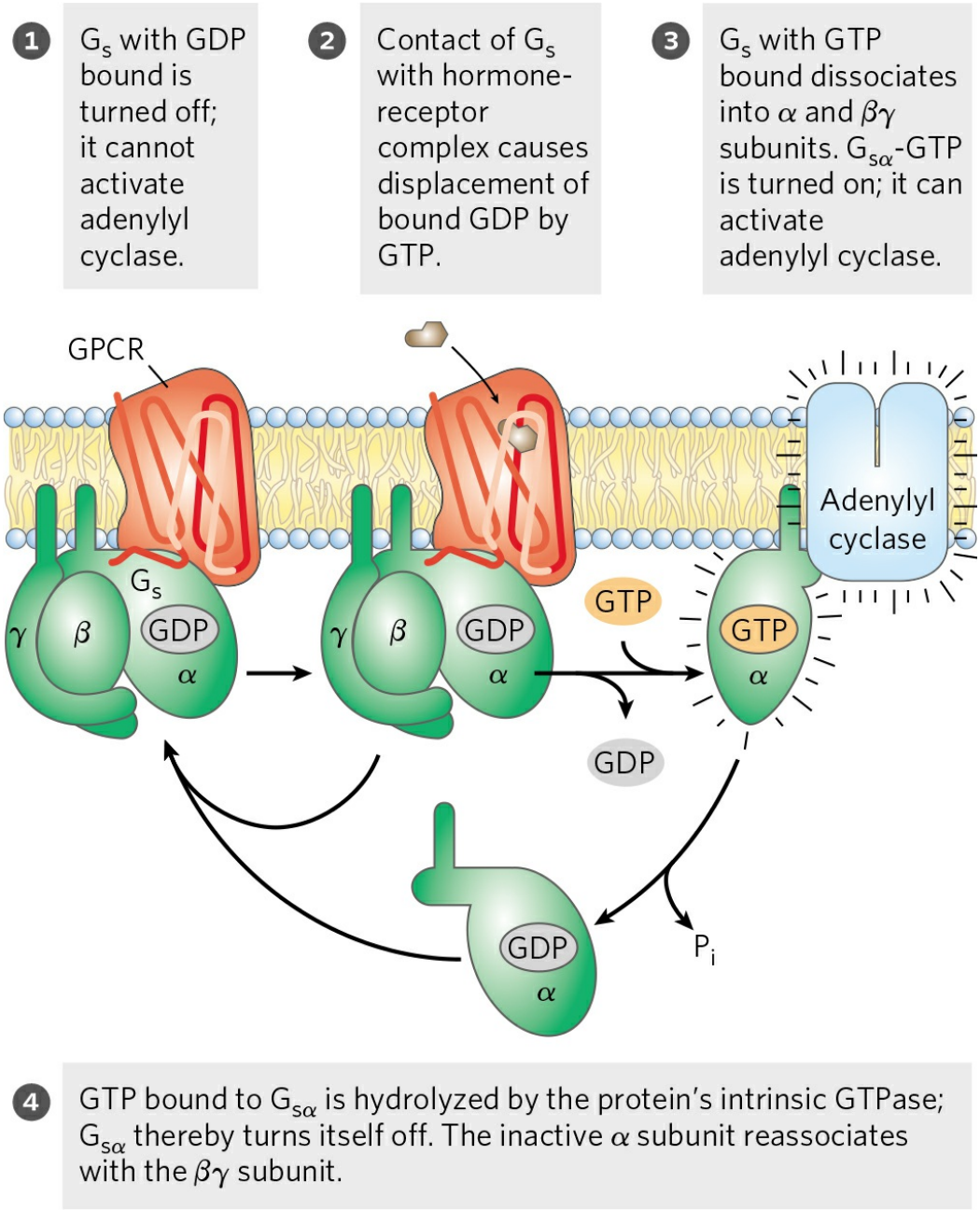


FIGURE 12-5 The GTPase switch. G proteins cycle between GDP-bound (off) and GTP-bound (on). The protein's intrinsic GTPase activity, in many cases stimulated by RGS proteins (*r*egulators of *G*-protein signaling; see Box 12-1), determines how quickly bound GTP is hydrolyzed to GDP and thus how long the G protein remains active.

The inactive form of PKA has two identical catalytic subunits (C) and two identical regulatory subunits (R) **(Fig. 12-6a)**. The tetrameric R_2C_2 complex is catalytically inactive, because an autoinhibitory domain of each R subunit occupies the substrate-binding cleft of each C subunit. Cyclic AMP is an allosteric activator of PKA. When cAMP binds to the R subunits, they undergo a conformational change that moves the

autoinhibitory domain of R out of the catalytic domain of C, and the R_2C_2 complex dissociates to yield two free, catalytically active C subunits. This same basic mechanism—displacement of an autoinhibitory domain—mediates the allosteric activation of many types of protein kinases by their second messengers (as in Figs 12-18 and 12-25, for example). The structure of the substrate-binding cleft in PKA is the prototype for all known protein kinases (Fig. 12-6b); certain residues in this cleft region have identical counterparts in all of the 544 protein kinases encoded in the human genome. The ATP-binding site of each catalytic subunit positions ATP perfectly for the transfer of its terminal (γ) phosphoryl group to the —OH in the side chain of a Ser or Thr residue in the target protein.

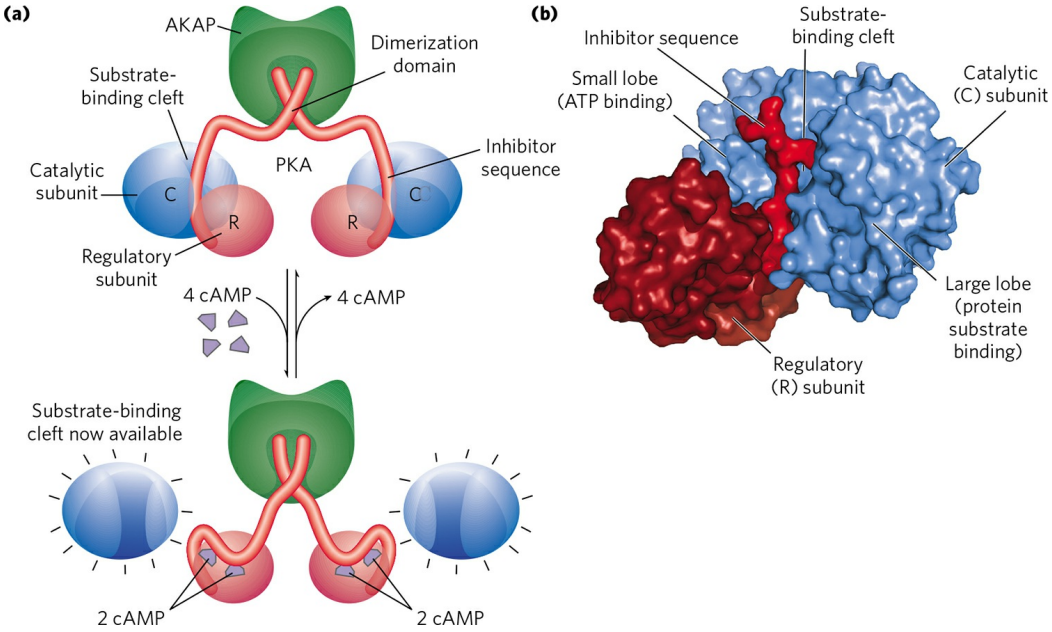


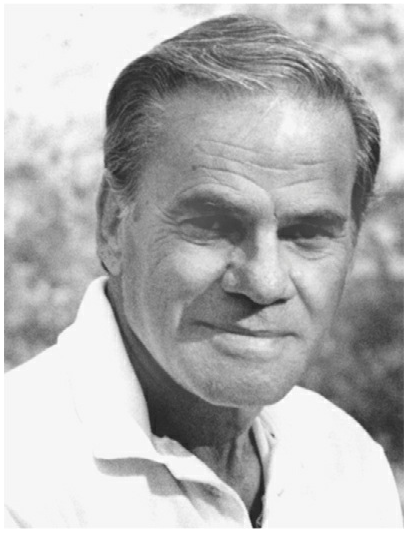
FIGURE 12-6 Activation of cAMP-dependent protein kinase (PKA). (a) When [cAMP] is low, the two identical regulatory subunits (R; red) associate with the two identical catalytic subunits (C). In this R_2C_2 complex, the inhibitor sequences of the R subunits lie in the substrate-binding cleft of the C subunits and prevent binding of protein substrates; the complex is therefore catalytically inactive. The amino-terminal sequences of the R subunits interact to form an R_2 dimer, the site of binding to an A kinase anchoring protein (AKAP), described later in the text. When [cAMP] rises in response to a hormonal signal, each R subunit binds two cAMP molecules and undergoes a dramatic reorganization that pulls its inhibitory sequence away from the C subunit, opening up the substrate-binding cleft and releasing each C subunit in its catalytically active form. **(b)** A crystal structure showing part of the R_2C_2 complex—one C subunit and part of one R subunit. The amino-terminal dimerization region of the R subunit is omitted for

simplicity. The small lobe of C contains the ATP-binding site, and the large lobe surrounds and defines the cleft where the protein substrate binds and undergoes phosphorylation at a Ser or Thr residue, with a phosphoryl group transferred from ATP. In this inactive form, the inhibitor sequence of R blocks the substrate-binding cleft of C, inactivating it.
[Source: (b) PDB ID 3FHI, C. Kim et al., *Science* 307:690, 2005.]

BOX 12-1 G Proteins: Binary Switches in Health and Disease

Alfred G. Gilman and Martin Rodbell discovered the critical roles of guanosine nucleotide–binding proteins (G proteins) in a wide variety of cellular processes, including sensory perception, signaling for cell division, growth and differentiation, intracellular movements of proteins and membrane vesicles, and protein synthesis. The human genome encodes nearly 200 of these proteins, which differ in size and subunit structure, intracellular location, and function. But all G proteins share a common feature: they can become activated and then, after a brief period, can inactivate themselves, thereby serving as molecular binary switches with built-in timers. This superfamily of proteins includes the trimeric G proteins involved in adrenergic signaling (G_s and G_i) and vision (transducin); small G proteins such as that involved in insulin signaling (Ras) and others that function in vesicle trafficking (ARF and Rab), transport into and out of the nucleus (Ran; see Fig. 27-44), and timing of the cell cycle (Rho); and several proteins involved in protein synthesis (initiation factor IF2 and elongation factors EF-Tu and EF-G; see Chapter 27). Many G proteins have covalently bound lipids, which give them an affinity for membranes and dictate their locations in the cell.

Alfred G. Gilman, 1941–2015
[Source: Shelly Katz/Liaison Agency/Getty Images.]



Martin Rodbell, 1925–1998
[Source: Courtesy Andrew M. Rodbell.]

All G proteins have the same core structure and use the same mechanism for switching between an inactive conformation, favored when GDP is bound, and an active conformation, favored when GTP is bound. We can use the Ras protein (~20 kDa), a minimal signaling unit, as a prototype for all members of this superfamily (Fig. 1).

In the GTP-bound conformation, the G protein exposes previously buried regions (called **switch I** and **switch II**) that interact with proteins downstream in the signaling pathway, until the G protein inactivates itself by hydrolyzing its bound GTP to GDP. The critical determinant of G-protein conformation is the *γ* phosphate of GTP, which interacts with a region called the **P loop** (*p*hosphate-binding; Fig. 2). In Ras, the *γ*

phosphate of GTP binds to a Lys residue in the P loop and to two critical residues, Thr^{35} in switch I and Gly^{60} in switch II, that hydrogen-bond with the oxygens of the γ phosphate of GTP. These hydrogen bonds act like a pair of springs holding the protein in its active conformation. When GTP is cleaved to GDP and P_i is released, these hydrogen bonds are lost; the protein then relaxes into its inactive conformation, burying the sites that, in its active state, interact with other partners. Ala^{146} hydrogen-bonds to the guanine oxygen, allowing GTP, but not ATP, to bind.

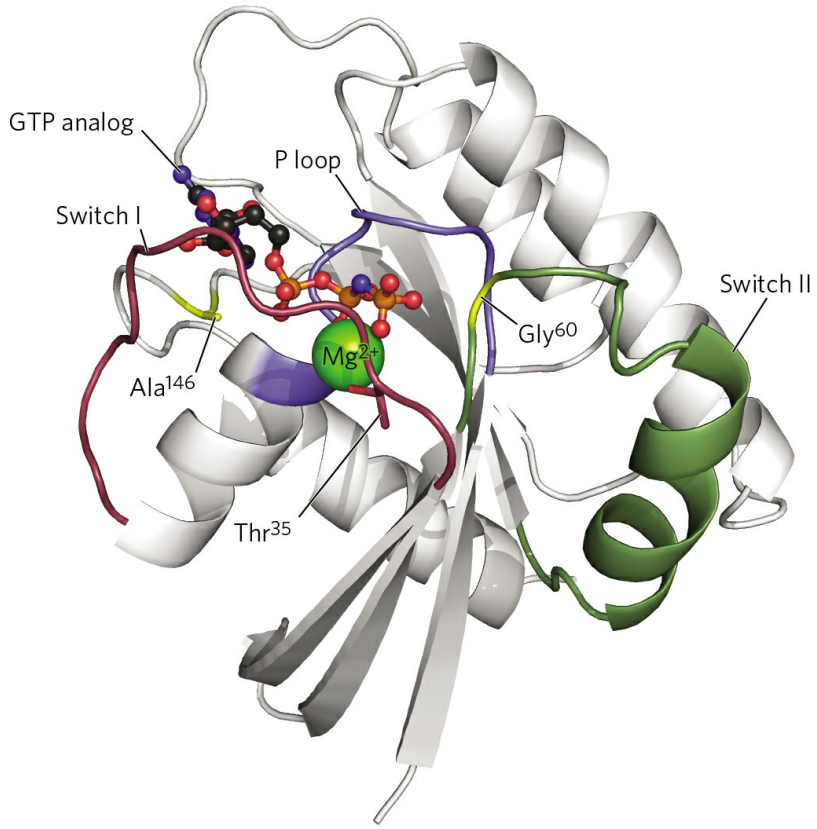


FIGURE 1 The Ras protein, the prototype for all G proteins. Mg^{2+}-GTP is held by critical residues in the phosphate-binding P loop (blue) and by Thr^{35} in the switch I (red) and Gly^{60} in the switch II (green) regions. Ala^{146} gives specificity for GTP over ATP. In the structure shown here, the nonhydrolyzable GTP analog Gpp(NH)p is in the GTP-binding site.
[Source: PDB ID 5P21, E. F. Pai et al., *EMBO J.* 9:2351, 1990.]

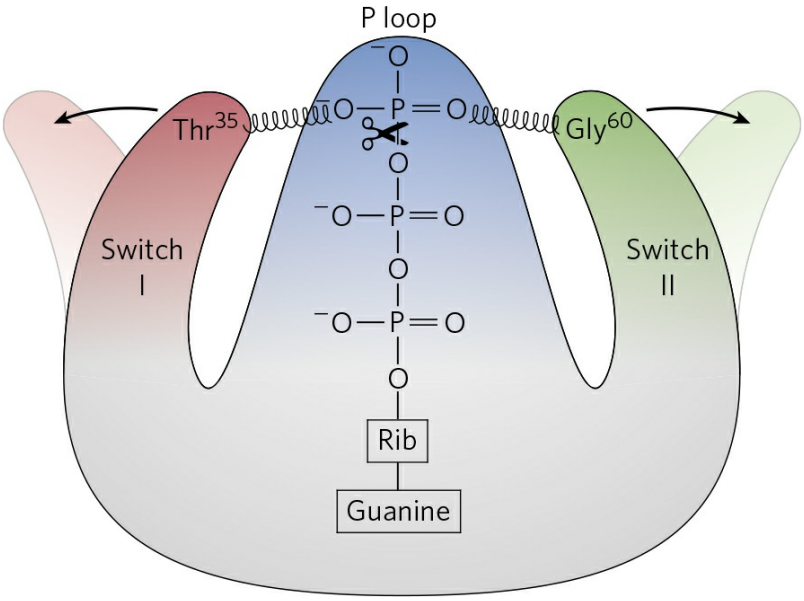


FIGURE 2 When bound GTP is hydrolyzed by the GTPase activities of Ras and its GTPase activator protein (GAP), loss of hydrogen bonds to Thr^{35} and Gly^{60} allows the switch I and switch II regions to relax into a conformation in which they are no longer available to interact with downstream targets.
[Source: Information from I. R. Vetter and A. Wittinghofer, *Science* 294:1299, 2001, Fig. 3.]

The intrinsic GTPase activity of most G proteins is very weak, but is increased up to 10^5-fold by **GTPase activator proteins (GAPs)**, also called, in the case of heterotrimeric G proteins, **regulators of G protein signaling (RGSs**; Fig. 3). GAPs (and RGSs) thus determine how long the switch remains on. They contribute a critical Arg residue that reaches into the G-protein GTPase active site and assists in catalysis. The intrinsically slow process of replacing bound GDP with GTP, switching the protein on, is catalyzed by **guanosine nucleotide–exchange factors (GEFs)** associated with the G protein (Fig. 3). The ligand-bound β-adrenergic receptor is one of many GEFs, and a broad range of proteins act as GAPs. Their combined effects set the level of GTP-bound G proteins, and thus the strength of the response to signals that arrive at the receptors.

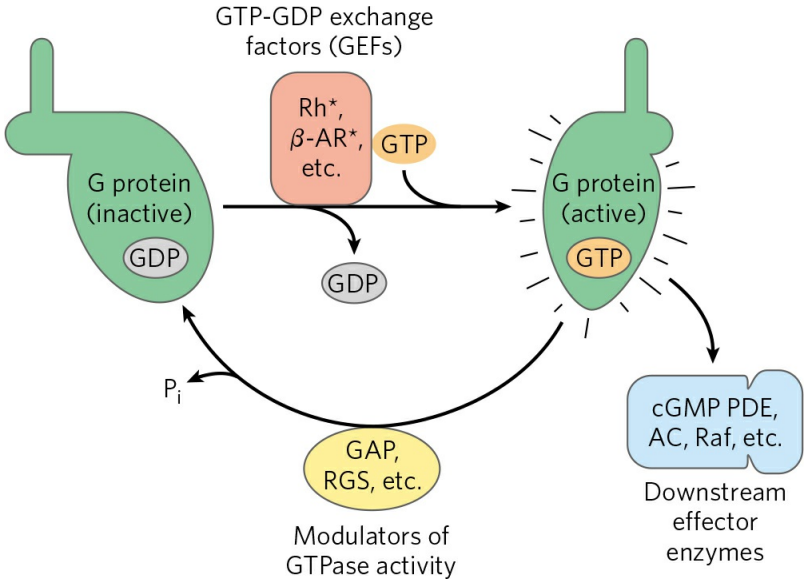


FIGURE 3 Many factors regulate the activity of G proteins (green). Inactive G proteins, both small G proteins such as Ras and heterotrimeric G proteins such as G_s, interact with upstream GTP-GDP exchange factors (red). Often these exchange factors are activated (*) receptors such as rhodopsin (Rh) and β-adrenergic receptors (AR). The G proteins are activated by GTP binding, and in the GTP-bound form, activate downstream effector enzymes (blue), such as cGMP phosphodiesterase (PDE), adenylyl cyclase (AC), and Raf. GTPase activator proteins (GAPs, in the case of small G proteins) and regulators of G protein signaling (RGSs) (yellow), by modulating the GTPase activity of G proteins, determine how long the G protein will remain active.

Because G proteins play crucial roles in so many signaling processes, it is not surprising that defects in G proteins lead to a variety of diseases. In about 25% of all human cancers (and in a much higher proportion of certain types of cancer), there is a mutation in a Ras protein—typically in one of the critical residues around the GTP-binding site or in the P loop—that virtually eliminates its GTPase activity. Once activated by GTP binding, this Ras protein remains constitutively active, promoting cell division in cells that should not divide. The tumor suppressor gene *NF1* encodes a GAP that enhances the GTPase activity of normal Ras. Mutations in *NF1* that result in a nonfunctioning GAP leave Ras with only its intrinsic GTPase activity, which is very weak (that is, has a very low turnover number); once activated by GTP binding, Ras stays active for an extended period, continuing to send the signal: divide.

Defective heterotrimeric G proteins can also lead to disease. Mutations in the gene that encodes the α subunit of G_s (which mediates changes in [cAMP] in response to hormonal stimuli) may result in a G_α that is permanently active or permanently inactive. "Activating" mutations generally occur in residues crucial to GTPase activity; they lead to a continuously elevated [cAMP], with significant downstream consequences, including undesirable cell proliferation. For example, such mutations are found in about 40% of pituitary tumors (adenomas). Individuals with "inactivating" mutations in G_α are unresponsive to hormones (such as thyroid hormone) that act through cAMP. Mutation in the gene for the transducin α subunit (T_α), which is involved in visual signaling, leads to a type of night blindness, apparently due to defective interaction between the activated T_α subunit and the phosphodiesterase of the rod outer segment (see Fig. 12-14). A sequence variation in the gene encoding the β subunit of a heterotrimeric G protein is commonly found in individuals with hypertension (high blood pressure), and this variant gene is suspected of involvement in obesity and atherosclerosis.

The pathogenic bacterium that causes cholera produces a toxin that targets a G protein, interfering with normal signaling in host cells. **Cholera toxin**, secreted by *Vibrio cholerae* in the intestine of an infected person, is a heterodimeric protein. Subunit B recognizes and binds to specific gangliosides on the surface of intestinal epithelial cells and provides a route for subunit A to enter these cells. After entry, subunit A is broken into two fragments, A1 and A2. A1 associates with the host cell's ADP-ribosylation factor ARF6, a small G protein, through residues in its switch I and switch II regions—which are accessible only when ARF6 is in its active (GTP-bound) form. This association with ARF6 activates A1, which catalyzes the transfer of ADP-ribose from NAD^+ to the critical Arg residue in the P loop of the α subunit of G_s (Fig. 4). ADP-ribosylation blocks the GTPase activity of G_s and thereby renders G_s permanently active. This results in continuous activation of the adenylyl cyclase of intestinal epithelial cells, chronically high [cAMP], and chronically active PKA. PKA phosphorylates the CFTR Cl^- channel (see Box 11-2) and a Na^+-H^+ exchanger in the intestinal epithelial cells. The resultant efflux of NaCl triggers massive water loss through the intestine as cells respond to the ensuing osmotic imbalance. Severe dehydration and electrolyte loss are

the major pathologies in cholera. These can be fatal in the absence of prompt rehydration therapy. ■

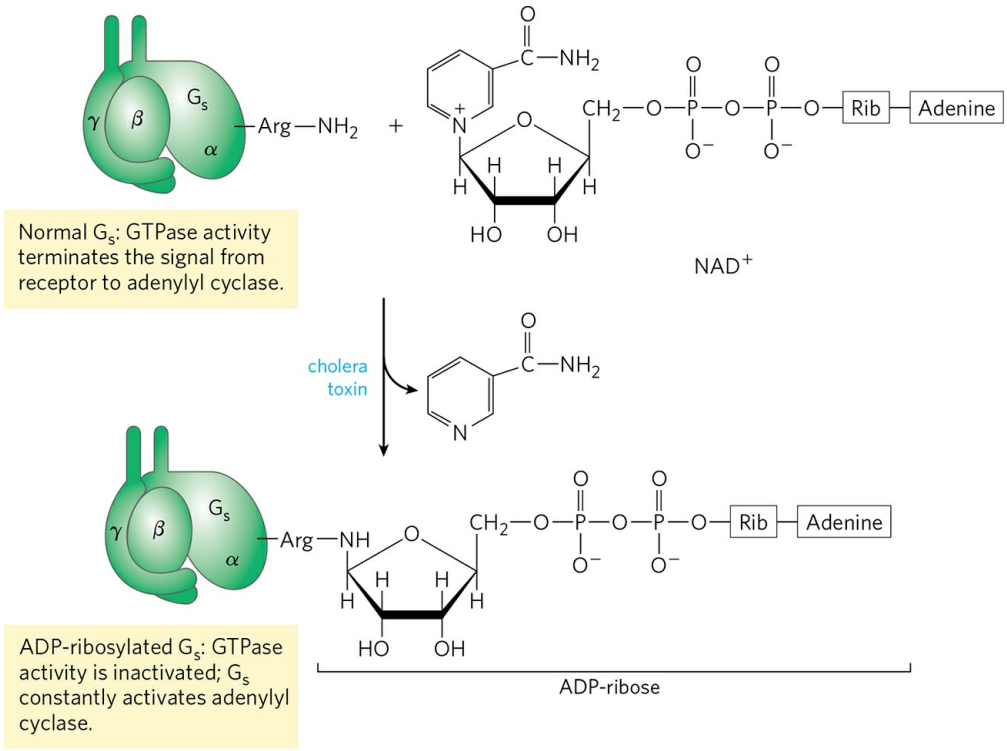


FIGURE 4 The bacterial toxin that causes cholera is an enzyme that catalyzes transfer of the ADP-ribose moiety of NAD^+ to an Arg residue of G_s. The G proteins thus modified fail to respond to normal hormonal stimuli. The pathology of cholera results from defective regulation of adenylyl cyclase and overproduction of cAMP.

As indicated in Figure 12-4a (step 6), PKA regulates many enzymes downstream in the signaling pathway. Although these downstream targets have diverse functions, they share a region of sequence similarity around the Ser or Thr residue that undergoes phosphorylation, a sequence that marks them for regulation by PKA (Table 12-2). The substrate-binding cleft of PKA recognizes these sequences and phosphorylates their Thr or Ser residue. Comparison of the sequences of various protein substrates for PKA has yielded the **consensus sequence**—the neighboring residues needed to mark a Ser or Thr residue for phosphorylation.

As in many signaling pathways, signal transduction by adenylyl cyclase entails several steps that *amplify* the original hormone signal **(Fig. 12-7)**. First, the binding of one hormone molecule to one receptor molecule catalytically activates many G_s molecules that associate with the activated receptor, one after the other. Next, by activating one molecule of

adenylyl cyclase, each active $G_{s\alpha}$ molecule stimulates the catalytic synthesis of *many* molecules of cAMP. The second messenger cAMP now activates PKA, each molecule of which catalyzes the phosphorylation of *many* molecules of the target protein—phosphorylase *b* kinase in Figure 12-7. This kinase activates glycogen phosphorylase *b*, which leads to the rapid mobilization of glucose from glycogen. The net effect of the cascade is amplification of the hormonal signal by several orders of magnitude, which accounts for the very low concentration of epinephrine (or any other hormone) required for hormone activity. This signaling pathway is also rapid: the signal leads to intracellular changes within milliseconds or even microseconds.

TABLE 12-2 Some Enzymes and Other Proteins Re
cAMP-Dependent Phosphorylation (by PK

Enzyme/protein	Sequence phosphorylated[a]	Pathway/ regulated
Glycogen synthase	RASCTSSS	Glycogen
Phosphorylase *b* kinase *α* subunit *β* subunit	VEFRRLSI RTKRSGSV	Glycogen
Pyruvate kinase (rat liver)	GVLRRASVAZL	Glycolysi
Pyruvate dehydrogenase complex (type L)	GYLRRASV	Pyruvate
Hormone-sensitive lipase	PMRRSV	Triacylgly mob acid
Phosphofructokinase-2/fructose 2,6-bisphosphatase	LQRRRGSSIPQ	Glycolysi
Tyrosine hydroxylase	FIGRRQSL	Synthesis dopa nore epin
Histone H1	AKRKASGPPVS	DNA con
Histone H2B	KKAKASRKESYSVYVYK	DNA con
Cardiac phospholamban	AIRRAST	Intracellu

(cardiac pump regulator)		
Protein phosphatase-1 inhibitor-1	IRRRRPTP	Protein de
PKA consensus sequence[b]	xR[RK]x[ST]B	Many

[a]The phosphorylated S or T residue is shown in red. All residues are given as their one-letter abbreviations (See Table 3-1).
[b] is any amino acid; B is any hydrophobic amino acid. See Box 3-2 for conventions used in displaying consensus sequences.

Several Mechanisms Cause Termination of the β-Adrenergic Response

To be useful, a signal-transducing system has to *turn off* after the hormonal or other stimulus has ended, and mechanisms for shutting off the signal are intrinsic to all signaling systems. Most systems also adapt to the continued presence of the signal by becoming less sensitive to it, by *desensitizing*. The β-adrenergic system illustrates both. Here, our focus is on termination.

The response to β-adrenergic stimulation will end when the concentration of epinephrine in the blood drops below the K_d for its receptor. The hormone then dissociates from the receptor, and the latter reassumes its inactive conformation, in which it can no longer activate G_s.

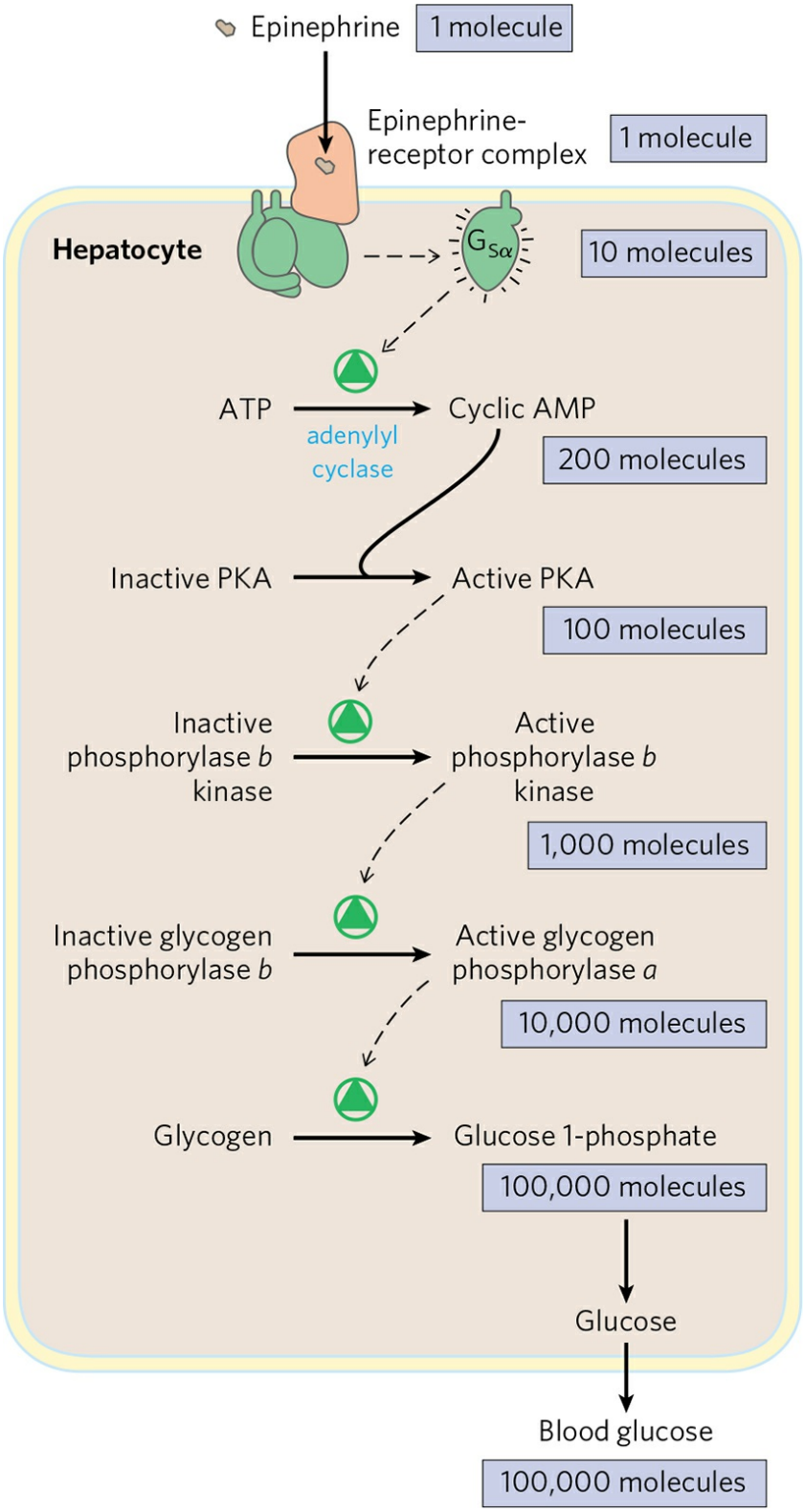


FIGURE 12-7 Epinephrine cascade. Epinephrine triggers a series of reactions in hepatocytes in which catalysts activate catalysts, resulting in great amplification of the original hormone signal. The numbers of molecules shown are simply to illustrate amplification and are almost certainly gross underestimates. Binding of one molecule of epinephrine to one β-adrenergic receptor on the cell surface activates many (possibly

hundreds of) G proteins, one after another, each of which goes on to activate a molecule of the enzyme adenylyl cyclase. Adenylyl cyclase acts catalytically, producing many molecules of cAMP for each activated adenylyl cyclase. (Because two molecules of cAMP are required to activate one PKA catalytic subunit, this step does not amplify the signal.)

A second means of ending the response is the hydrolysis of GTP bound to the G_α subunit, catalyzed by the intrinsic GTPase activity of the G protein. Conversion of bound GTP to GDP favors the return of G_α to the conformation in which it binds the $G_{\beta\gamma}$ subunits—the conformation in which the G protein is unable to interact with or stimulate adenylyl cyclase. This ends the production of cAMP. The rate of inactivation of G_s depends on the GTPase activity, which for G_α alone is very feeble. However, GTPase activator proteins (GAPs) strongly stimulate this GTPase activity, causing more rapid inactivation of the G protein (see Box 12-1). GAPs can themselves be regulated by other factors, providing a fine-tuning of the response to *β*-adrenergic stimulation. A third mechanism for terminating the response is to remove the second messenger: cAMP is hydrolyzed to 5′-AMP (not active as a second messenger) by **cyclic nucleotide phosphodiesterase** (Fig. 12-4a, step 7; 12-4b).

Finally, at the end of the signaling pathway, the metabolic effects that result from enzyme phosphorylation are reversed by the action of phosphoprotein phosphatases, which hydrolyze phosphorylated Ser, Thr, or Tyr residues, releasing inorganic phosphate (P_i). About 150 genes in the human genome encode phosphoprotein phosphatases, fewer than the number (544) encoding protein kinases, reflecting the relative promiscuity of the phosphoprotein phosphatase. A single phosphoprotein phosphatase (PP1) dephosphorylates some 200 different phosphoprotein targets. Some phosphatases are known to be regulated; others may act constitutively. When [cAMP] drops and PKA returns to its inactive form (step 7 in Fig. 12-4a), the balance between phosphorylation and dephosphorylation is tipped toward dephosphorylation by these phosphatases.

The *β*-Adrenergic Receptor Is Desensitized by Phosphorylation and by Association with Arrestin

The mechanisms for signal termination described above take effect when the stimulus ends. A different mechanism, desensitization, damps the response *even while the signal persists*. Desensitization of the *β*-adrenergic

receptor is mediated by a protein kinase that phosphorylates the receptor on the intracellular domain that normally interacts with G_s **(Fig. 12-8)**. When the receptor remains occupied with epinephrine, ***β*-adrenergic receptor kinase**, or ***β*ARK** (also commonly called **GRK2**; see below), phosphorylates several Ser residues near the receptor's carboxyl terminus, which is on the cytoplasmic side of the plasma membrane. PKA, activated by the rise in [cAMP], phosphorylates, and thereby activates, *β*ARK. *β*ARK is then drawn to the plasma membrane by its association with the $G_{s\beta\gamma}$ subunits and is thus positioned to phosphorylate the receptor. Receptor phosphorylation creates a binding site for the protein ***β*-arrestin**, or ***β*arr** (also called arrestin 2), and binding of *β*-arrestin blocks the sites in the receptor that interact with the G protein **(Fig. 12-9)**. The binding of *β*-arrestin also facilitates the sequestration of receptor molecules, their removal from the plasma membrane by endocytosis into small intracellular vesicles (endosomes). The arrestin-receptor complex recruits clathrin and other proteins involved in vesicle formation (see Fig. 27-27), which initiate membrane invagination, leading to the formation of endosomes containing the adrenergic receptor. In this state, the receptors are inaccessible to epinephrine and therefore inactive. These receptor molecules are eventually dephosphorylated and returned to the plasma membrane, completing the circuit and resensitizing the system to epinephrine. *β*-Adrenergic receptor kinase is a member of a family of **G protein–coupled receptor kinases (GRKs)**, all of which phosphorylate GPCRs on their carboxyl-terminal cytoplasmic domains and play roles similar to that of *β*ARK in desensitization and resensitization of their receptors. At least five different GRKs and four different arrestins are encoded in the human genome; each GRK is capable of desensitizing a particular subset of GPCRs, and each arrestin can interact with many different types of phosphorylated receptors.

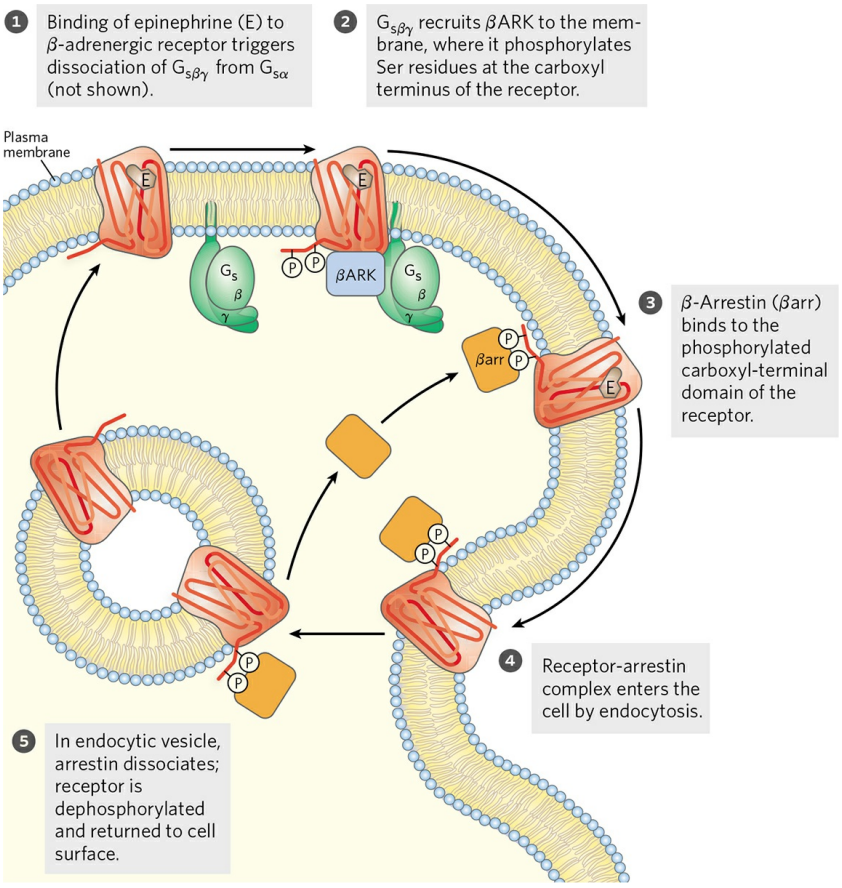


FIGURE 12-8 Desensitization of the *β*-adrenergic receptor in the continued presence of epinephrine. This process is mediated by two proteins: *β*-adrenergic protein kinase (*β*ARK) and *β*-arrestin (*β*arr; also known as arrestin 2). Not shown here is the phosphorylation and activation of *β*ARK by PKA. PKA is activated by the rise in [cAMP] in response to the initial signal, epinephrine.

The receptor-arrestin complex has another important role: it initiates signaling by a different pathway, the MAPK cascade described below. Thus, acting through a single GPCR, epinephrine triggers two distinct signaling pathways. The two pathways, one triggered by the receptor's interaction with a G protein and the other by its interaction with arrestin, can be differentially affected by the agonist; in some cases, one agonist favors the G-protein pathway and another favors the arrestin pathway. This bias is an important consideration in the development of a medication that acts through a GPCR. For example, the most addictive of the opioid drugs of abuse act more strongly through G-protein signaling than through arrestin. An ideal opioid pain medication would act through the branch of the pathway that has therapeutic effects and not through the pathway that leads to addiction. ■

Cyclic AMP Acts as a Second Messenger for Many Regulatory Molecules

Epinephrine is just one of many hormones, growth factors, and other regulatory molecules that act by changing the intracellular [cAMP] and thus the activity of PKA (Table 12-3). For example, glucagon binds to its receptors in the plasma membrane of adipocytes, activating (via a G_s protein) adenylyl cyclase. PKA, stimulated by the resulting rise in [cAMP], phosphorylates and activates two proteins critical to the mobilization of the fatty acids of stored fats (see Fig. 17-3). Similarly, the peptide hormone ACTH (adrenocorticotropic hormone, also called corticotropin), produced by the anterior pituitary, binds to specific receptors in the adrenal cortex, activating adenylyl cyclase and raising the intracellular [cAMP]. PKA then phosphorylates and activates several of the enzymes required for the synthesis of cortisol and other steroid hormones. In many cell types, the catalytic subunit of PKA can also move into the nucleus, where it phosphorylates the **cAMP response element binding protein (CREB)**, which alters the expression of specific genes regulated by cAMP.

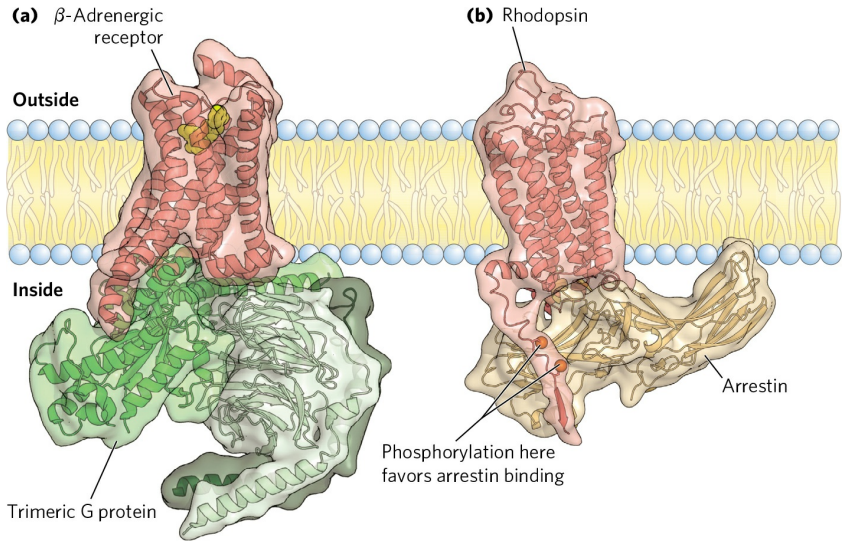


FIGURE 12-9 Mutual exclusion of trimeric G protein and arrestin in their interaction with a GPCR. (a) The complex of the *β*-adrenergic receptor with its trimeric G protein, G_s. **(b)** The complex of *β*-arrestin with the *β*-adrenergic receptor has not yet been solved, but the complex with another closely similar GCPR, visual rhodopsin, has, as shown here. (Rhodopsin is discussed later in the chapter.) Comparison of the two structures makes it clear that the binding of arrestin blocks the binding of the G protein and so prevents further activation of G proteins, effectively ending the response to the initial signal

(epinephrine). [Sources: (a) PDB ID 3SN6, S. G. F. Rasmussen et al., *Nature* 477:549, 2011, Fig. 2c. (b) PDB ID 4ZWJ, Y. Kang et al., *Nature* 523:561, 2015, Fig. 2b.]

TABLE 12-3 Some Signals That Use cAMP as Second Messenger

Corticotropin (ACTH)
Corticotropin-releasing hormone (CRH)
Dopamine [D_1, D_2]
Epinephrine (β-adrenergic)
Follicle-stimulating hormone (FSH)
Glucagon
Histamine [H_2]
Luteinizing hormone (LH)
Melanocyte-stimulating hormone (MSH)
Odorants (many)
Parathyroid hormone
Prostaglandins E_1, E_2 (PGE_1, PGE_2)
Serotonin [5-HT_1, 5-HT_4]
Somatostatin
Tastants (sweet, bitter)
Thyroid-stimulating hormone (TSH)

Note: Receptor subtypes in square brackets. Subtypes may have different transduction mechanisms. For example, serotonin is detected in some tissues by receptor subtypes 5-HT_1 and 5-HT_4, which act through adenylyl cyclase and cAMP, and in other tissues by receptor subtypes 5-HT_2, acting through the phospholipase C-IP_3 mechanism (see Table 12-4).

Some hormones act by *inhibiting* adenylyl cyclase, thus *lowering* [cAMP] and *suppressing* protein phosphorylation. For example, the binding of somatostatin to its receptor in the pancreas leads to activation of

an **inhibitory G protein**, or **G_i**, structurally homologous to G_s, that inhibits adenylyl cyclase and lowers [cAMP]. In this way, somatostatin inhibits the secretion of several hormones, including glucagon. In adipose tissue, prostaglandin E_2 (PGE_2; see Fig. 10-17) inhibits adenylyl cyclase, thus lowering [cAMP] and slowing the mobilization of lipid reserves triggered by epinephrine and glucagon. In certain other tissues, PGE_2 stimulates cAMP synthesis: its receptors are coupled to adenylyl cyclase through a stimulatory G protein, G_s. In tissues with α_2-adrenergic receptors, epinephrine lowers [cAMP]; in this case, the receptors are coupled to adenylyl cyclase through an inhibitory G protein, G_i. In short, an extracellular signal such as epinephrine or PGE_2 can have different effects on different tissues or cell types, depending on three factors: the type of receptor in the tissue, the type of G protein (G_s or G_i) with which the receptor is coupled, and the set of PKA target enzymes in the cell. By summing the influences that tend to increase and decrease [cAMP], a cell achieves the integration of signals that is a general feature of signal-transducing mechanisms (Fig. 12-1e).

Another factor that explains how so many types of signals can be mediated by a single second messenger (cAMP) is the confinement of the signaling process to a specific region of the cell by **adaptor proteins**—noncatalytic proteins that hold together other protein molecules that function in concert (further described below). **AKAPs (A kinase anchoring proteins)** have multiple distinct protein-binding domains; they are multivalent adaptor proteins. One domain binds to the R subunits of PKA (see Fig. 12-6a) and another binds to a specific structure in the cell, confining the PKA to the vicinity of that structure. For example, specific AKAPs bind PKA to microtubules, actin filaments, ion channels, mitochondria, or the nucleus. Different types of cells have different complements of AKAPs, so cAMP might stimulate phosphorylation of mitochondrial proteins in one cell and phosphorylation of actin filaments in another. In some cases, an AKAP connects PKA with the enzyme that triggers PKA activation (adenylyl cyclase) or terminates PKA action (cAMP phosphodiesterase or phosphoprotein phosphatase) **(Fig. 12-10)**. The very close proximity of these activating and inactivating enzymes presumably achieves a highly localized, and very brief, response.

As is now clear, to fully understand cellular signaling, researchers need tools precise enough to detect and study where signaling processes take place at the subcellular level and when they take place in real time. In

studies of the intracellular localization of biochemical changes, biochemistry meets cell biology, and techniques that cross this boundary have become essential in understanding signaling pathways. Fluorescent probes have found wide application in signaling studies. Labeling of functional proteins with a fluorescent tag such as the green fluorescent protein (GFP) reveals their location within the cell (see Fig. 9-16). Changes in the state of association of two proteins (such as the R and C subunits of PKA) can be seen by measuring the nonradiative transfer of energy between fluorescent probes attached to each protein, a technique called fluorescence resonance energy transfer (FRET; Box 12-2).

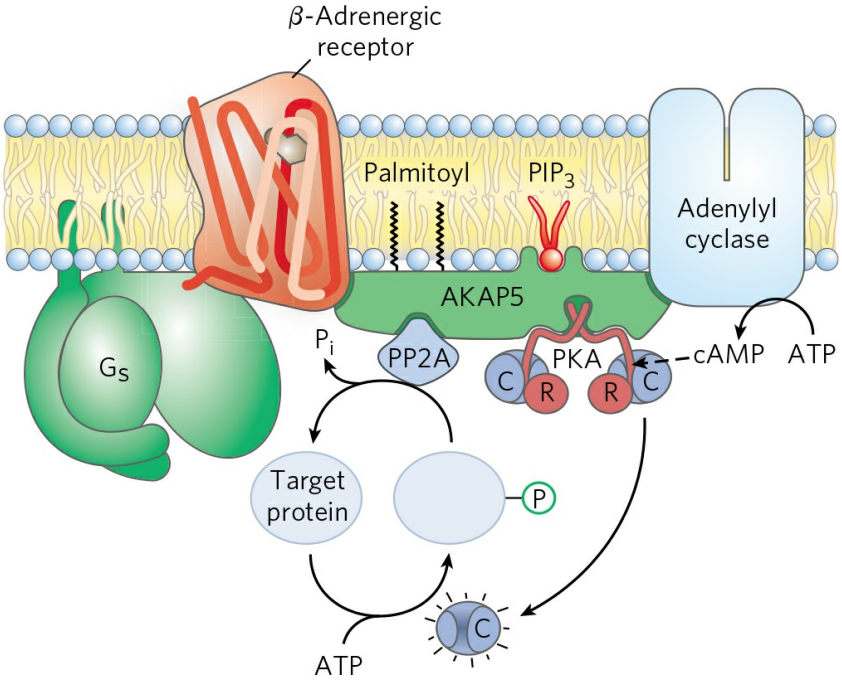
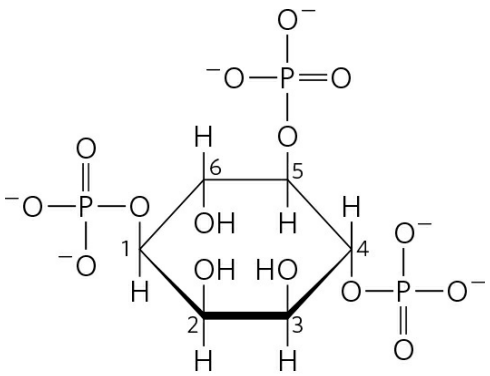


FIGURE 12-10 Nucleation of supramolecular complexes by A kinase anchoring proteins (AKAPs). AKAP5 is one of a family of proteins that act as multivalent scaffolds, holding PKA catalytic subunits—through interaction of the AKAP with the PKA regulatory subunits—in proximity to a particular region or structure in the cell. AKAP5 is targeted to rafts in the cytoplasmic face of the plasma membrane by two covalently attached palmitoyl groups and a site that binds phosphatidylinositol 3,4,5-trisphosphate (PIP_3) in the membrane. AKAP5 also has binding sites for the β-adrenergic receptor, adenylyl cyclase, PKA, and a phosphoprotein phosphatase (PP2A), bringing them all together in the plane of the membrane. When epinephrine binds to the β-adrenergic receptor, adenylyl cyclase produces cAMP, which reaches the nearby PKA quickly and with very little dilution. PKA phosphorylates its target protein, altering its activity, until the phosphoprotein phosphatase removes the phosphoryl group and returns

the target protein to its prestimulus state. The AKAPs in this and other cases bring about a high local concentration of enzymes and second messengers, so that the signaling circuit remains highly localized and the duration of the signal is limited.

Diacylglycerol, Inositol Trisphosphate, and Ca^{2+} Have Related Roles as Second Messengers

A second broad class of GPCRs are coupled through a G protein to a plasma membrane **phospholipase C (PLC)** that catalyzes cleavage of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate, or PIP_2 (see Fig. 10-15). When one of the hormones that acts by this mechanism (Table 12-4) binds its specific receptor in the plasma membrane (**Fig. 12-11**, step 1), the receptor-hormone complex catalyzes GTP-GDP exchange on an associated G protein, $\mathbf{G_q}$ (step 2), activating it in much the same way that the β-adrenergic receptor activates G_s (Fig. 12-4). The activated G_q activates the PIP_2-specific PLC (Fig. 12-11, step 3), which catalyzes the production of two potent second messengers (step 4), **diacylglycerol** and **inositol 1,4,5-trisphosphate**, or $\mathbf{IP_3}$ (not to be confused with PIP_3, p. 463).



Inositol 1,4,5-trisphosphate (IP_3)

Inositol trisphosphate, a water-soluble compound, diffuses from the plasma membrane to the endoplasmic reticulum (ER), where it binds to specific IP_3-gated Ca^{2+} channels, causing them to open. The action of the SERCA pump (p. 414) ensures that $[Ca^{2+}]$ in the ER is orders of magnitude higher than that in the cytosol, so when these gated Ca^{2+} channels open, Ca^{2+} rushes into the cytosol (Fig. 12-11, step 5), and the cytosolic $[Ca^{2+}]$ rises sharply to about 10^{-6} M. One effect of elevated $[Ca^{2+}]$ is the activation of **protein kinase C** (**PKC**; *C* for Ca^{2+}).

Diacylglycerol cooperates with Ca^{2+} in activating PKC, thus also acting as a second messenger (step 6). Activation involves the movement of a PKC domain (the pseudosubstrate domain) away from its location in the substrate-binding region of the enzyme, allowing the enzyme to bind and phosphorylate proteins that contain a PKC consensus sequence—Ser or Thr residues embedded in an amino acid sequence recognized by PKC (step 7). There are several isozymes of PKC, each with a characteristic tissue distribution, target protein specificity, and role. Their targets include cytoskeletal proteins, enzymes, and nuclear proteins that regulate gene expression. Taken together, this family of enzymes has a wide range of cellular actions, affecting neuronal and immune function and the regulation of cell division. Compounds that lead to overexpression of PKC or increase its activity to abnormal levels act as tumor promoters; animals exposed to these substances have increased rates of cancer.

TABLE 12-4 Some Signals That Act through Phospholipase C, IP_3, and Ca^{2+}

Acetylcholine [muscarinic M_1]	Gastrin-releasing peptide	Platelet-derived growth factor (PDGF)
α_1-Adrenergic agonists	Glutamate	Serotonin [5-HT_2]
Angiogenin	Gonadotropin-releasing hormone (GRH)	Thyrotropin-releasing hormone (TRH)
Angiotensin II	Histamine [H_1]	Vasopressin
ATP [P_{2x}, P_{2y}]	Light (*Drosophila*)	
Auxin	Oxytocin	

Note: Receptor subtypes are in square brackets; see footnote to Table 12-3.

BOX 12-2 METHODS FRET: Biochemistry Visualized in a Living Cell

Fluorescent probes are commonly used to detect rapid biochemical changes in single living cells. They can be designed to give an essentially instantaneous report (within nanoseconds) on the changes in intracellular concentration of a second messenger or in the activity of a protein kinase. Furthermore, fluorescence microscopy has sufficient resolution to reveal where in the cell such changes are occurring. In one widely used procedure, the fluorescent probes are derived from a naturally occurring fluorescent protein, the **green fluorescent protein (GFP)**, described in Chapter 9 (see Fig. 9-16), and variants with different fluorescence spectra, produced by genetic engineering or obtained from various marine coelenterates. For example, in the yellow fluorescent protein (YFP), Ala^{206} in GFP is replaced by a Lys residue, changing the wavelength of light absorption and fluorescence. Other variants of GFP fluoresce blue (BFP) or cyan (CFP) light, and a related protein (mRFP1) fluoresces red light (Fig. 1). GFP and its variants are compact structures that retain their ability to fold into their native β-barrel conformation even when fused with another protein. These fluorescent hybrid proteins act as spectroscopic rulers for measuring distances between interacting proteins within a cell and, indirectly, as measures of local concentrations of compounds that change the distance between two proteins.

An excited fluorescent molecule such as GFP or YFP can dispose of the energy from the absorbed photon in either of two ways: (1) by fluorescence, emitting a photon of slightly longer wavelength (lower energy) than the exciting light, or (2) by nonradiative **fluorescence resonance energy transfer (FRET)**, in which the energy of the excited molecule (the donor) passes directly to a nearby molecule (the acceptor) *without emission of a photon*, exciting the acceptor (Fig. 2). The acceptor can now decay to its ground state by fluorescence; the emitted photon has a longer wavelength (lower energy) than both the original exciting light and the fluorescence emission of the donor. This second mode of decay (FRET) is possible only when donor and acceptor are close to each other (within 1 to 50 Å); the efficiency of FRET is inversely proportional to the *sixth power* of the distance between donor and acceptor. Thus very small changes in the distance between donor and acceptor register as very large changes in FRET, measured as the fluorescence of the acceptor molecule when the donor is excited. With sufficiently sensitive light detectors, this fluorescence signal can be located to specific regions of a single, living cell.

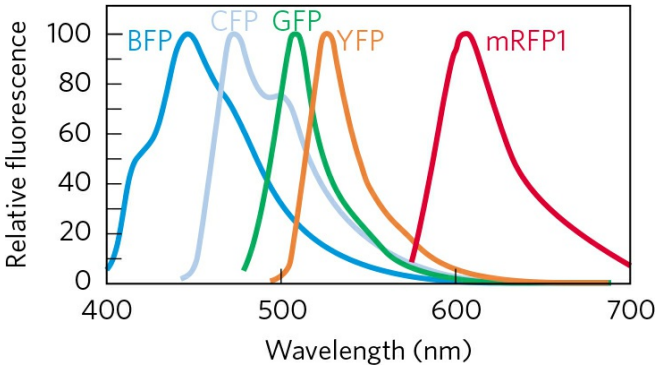


FIGURE 1 Emission spectra of some GFP variants.

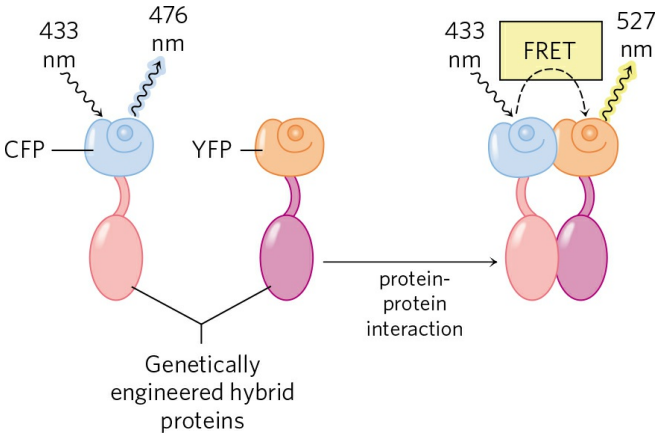


FIGURE 2 When the donor protein (CFP) is excited with monochromatic light of wavelength 433 nm, it emits fluorescent light at 476 nm (left). When the (red) protein fused with CFP interacts with the (purple) protein fused with YFP, that interaction brings CFP and YFP close enough to allow fluorescence resonance energy transfer (FRET) between them. Now, when CFP absorbs light of 433 nm, instead of fluorescing at 476 nm, it transfers energy directly to YFP, which then fluoresces at its characteristic emission wavelength, 527 nm. The ratio of light emission at 527 and 476 nm is therefore a measure of the extent of interaction between the red and purple proteins.

FRET has been used to measure [cAMP] in living cells. The gene for BFP is fused with that for the regulatory subunit (R) of cAMP-dependent protein kinase (PKA), and the gene for GFP is fused with that for the catalytic subunit (C) (Fig. 3). When these two hybrid proteins are expressed in a cell, BFP (donor; excitation at 380 nm, emission at 460 nm) and GFP (acceptor; excitation at 475 nm, emission at 545 nm) in the inactive PKA (R_2C_2 tetramer) are close enough to undergo FRET. Wherever in the cell [cAMP] increases, the R_2C_2 complex dissociates into R_2 and 2 C and the FRET signal is lost,

because donor and acceptor are now too far apart for efficient FRET. Viewed in the fluorescence microscope, the region of higher [cAMP] has a minimal GFP signal and higher BFP signal. Measuring the ratio of emission at 460 nm and 545 nm gives a sensitive measure of the change in [cAMP]. By determining this ratio for all regions of the cell, the investigator can generate a false color image of the cell in which the ratio, or relative [cAMP], is represented by the intensity of the color. Images recorded at timed intervals reveal changes in [cAMP] over time.

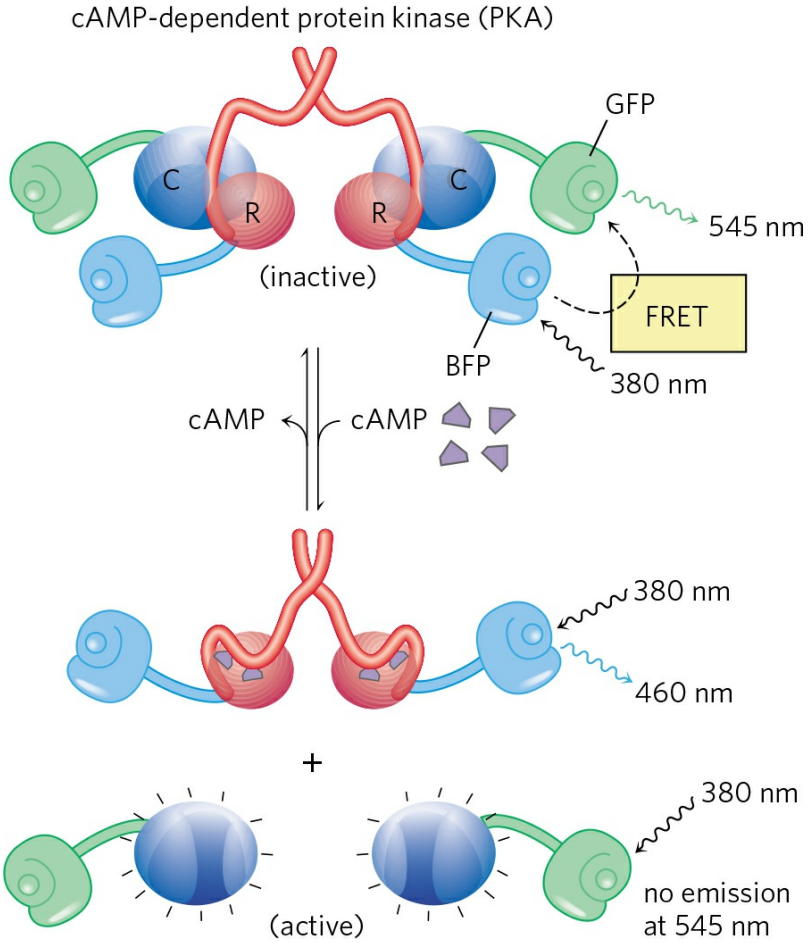


FIGURE 3 Measuring [cAMP] with FRET. Gene fusion creates hybrid proteins that exhibit FRET when the PKA regulatory (R) and catalytic (C) subunits are associated (low [cAMP]). When [cAMP] rises, the subunits dissociate and FRET ceases. The ratio of emission at 460 nm (dissociated) and 545 nm (complexed) thus offers a sensitive measure of [cAMP].

A variation of this technology has been used to measure the activity of PKA in a living cell (Fig. 4). Researchers create a phosphorylation target for PKA by producing a hybrid protein containing four elements: YFP (acceptor); a short peptide with a Ser residue surrounded by the

consensus sequence for PKA; a Ⓟ–Ser-binding domain (called 14-3-3); and CFP (donor). When the Ser residue is not phosphorylated, 14-3-3 has no affinity for the Ser residue and the hybrid protein exists in an extended form, with the donor and acceptor too far apart to generate a FRET signal. Wherever PKA is active in the cell, it phosphorylates the Ser residue of the hybrid protein, and 14-3-3 binds to the Ⓟ–Ser. In doing so, it draws YFP and CFP together and a FRET signal is detected with the fluorescence microscope, revealing the presence of active PKA.

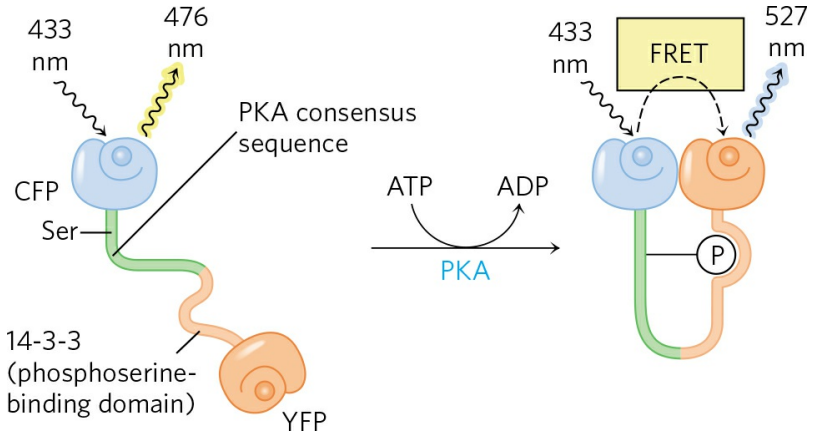


FIGURE 4 Measuring the activity of PKA with FRET. An engineered protein links YFP and CFP via a peptide that contains (1) a Ser residue surrounded by the consensus sequence for phosphorylation by PKA and (2) the 14-3-3 Ⓟ–Ser-binding domain. Active PKA phosphorylates the Ser residue, which docks with the 14-3-3 binding domain, bringing the fluorescence proteins close enough to allow FRET, revealing the presence of active PKA.

Calcium Is a Second Messenger That Is Localized in Space and Time

There are many variations on this basic scheme for Ca^{2+} signaling. In many cell types that respond to extracellular signals, Ca^{2+} serves as a second messenger that triggers intracellular responses, such as exocytosis in neurons and endocrine cells, contraction in muscle, and cytoskeletal rearrangements during amoeboid movement. In unstimulated cells, cytosolic [Ca^{2+}] is kept very low ($<10^{-7}$ M) by the action of Ca^{2+} pumps in the ER, mitochondria, and plasma membrane (as further discussed below). Hormonal, neural, or other stimuli cause either an influx of Ca^{2+}

into the cell through specific Ca^{2+} channels in the plasma membrane or the release of sequestered Ca^{2+} from the ER or mitochondria, in either case raising the cytosolic [Ca^{2+}] and triggering a cellular response.

Changes in intracellular [Ca^{2+}] are detected by Ca^{2+}-binding proteins that regulate a variety of Ca^{2+}-dependent enzymes. **Calmodulin** (**CaM**; M_r 17,000) is an acidic protein with four high-affinity Ca^{2+}-binding sites. When intracellular [Ca^{2+}] rises to about 10^{+6} M (1 μM), the binding of Ca^{2+} to calmodulin drives a conformational change in the protein **(Fig. 12-12a)**. Calmodulin associates with a variety of proteins and, in its Ca^{2+}-bound state, modulates their activities (Fig. 12-12b). It is a member of a family of Ca^{2+}-binding proteins that also includes troponin (see Fig. 5-32), which triggers skeletal muscle contraction in response to increased [Ca^{2+}]. Members of this family share a characteristic Ca^{2+}-binding structure, the EF hand (Fig. 12-12c).

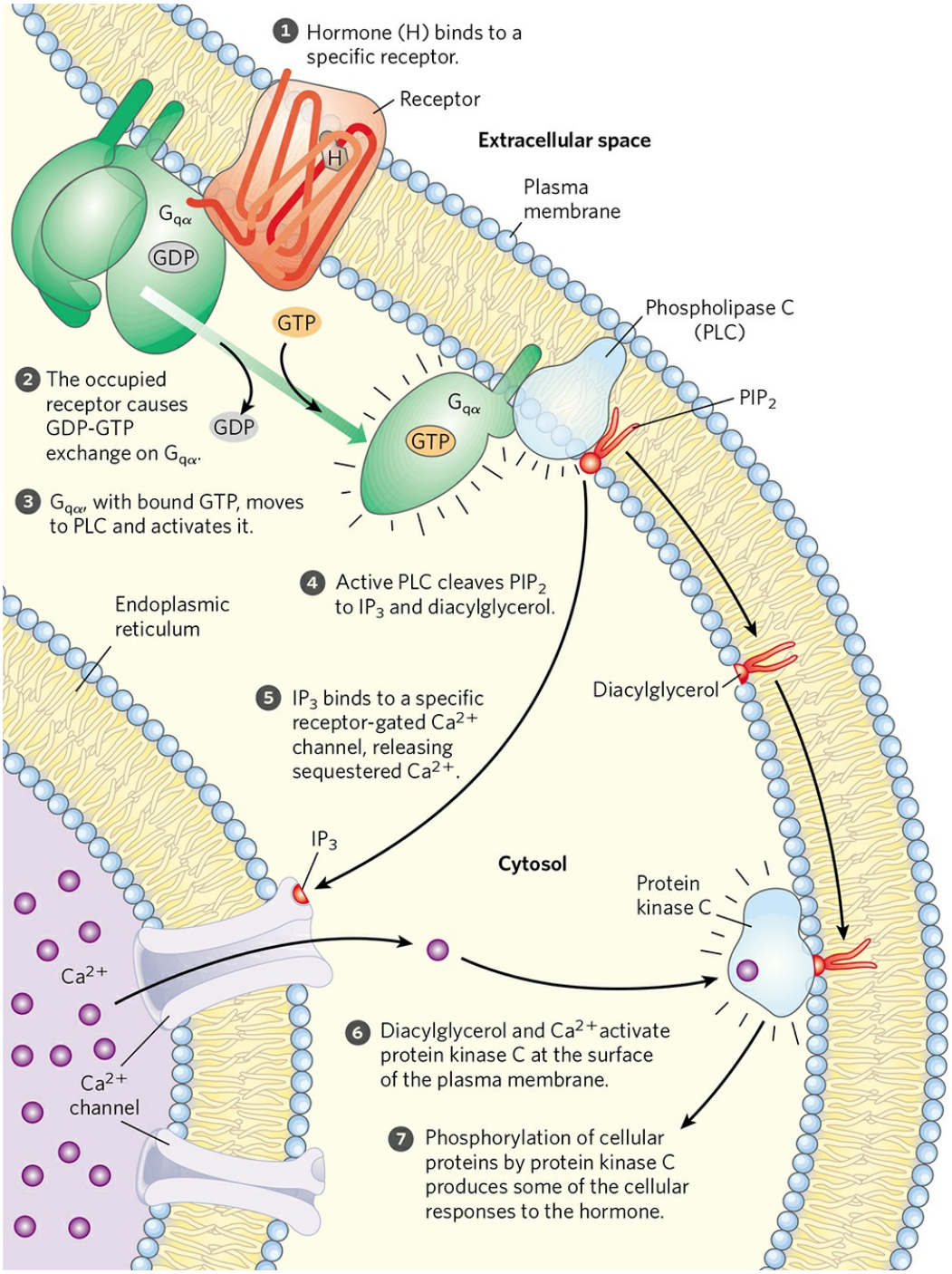


FIGURE 12-11 Hormone-activated phospholipase C and IP3. Two intracellular second messengers are produced in the hormone-sensitive phosphatidylinositol system: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol are cleaved from phosphatidylinositol 4,5-bisphosphate (PIP_2). Both contribute to the activation of protein kinase C. By raising cytosolic [Ca^{2+}], IP_3 also activates other Ca^{2+}-dependent enzymes; thus Ca^{2+} also acts as a second messenger.

Calmodulin is an integral subunit of the **Ca^{2+}/calmodulin-dependent protein kinases** (**CaM kinases**, types I through IV). When intracellular [Ca^{2+}] increases in response to a stimulus, calmodulin binds Ca^{2+}, undergoes a change in conformation, and activates the CaM kinase. The kinase then phosphorylates target enzymes, regulating their activities. Calmodulin is also a regulatory subunit of phosphorylase *b* kinase of muscle, which is activated by Ca^{2+}. Thus Ca^{2+} triggers ATP-requiring muscle contractions while also activating glycogen breakdown, providing fuel for ATP synthesis. Many other enzymes are also known to be modulated by Ca^{2+} through calmodulin (Table 12-5). The activity of the second messenger Ca^{2+}, like that of cAMP, can be spatially restricted; after its release triggers a local response, Ca^{2+} is generally removed before it can diffuse to distant parts of the cell.

Commonly, Ca^{2+} level does not simply rise and then fall, but rather oscillates with a period of a few seconds **(Fig. 12-13)**—even when the extracellular concentration of the triggering hormone remains constant. The mechanism underlying [Ca^{2+}] oscillations presumably entails feedback regulation by Ca^{2+} on some part of the Ca^{2+}-release process. Whatever the mechanism, the effect is that one kind of signal (hormone concentration, for example) is converted into another (frequency and amplitude of intracellular [Ca^{2+}] "spikes"). The Ca^{2+} signal diminishes as Ca^{2+} diffuses away from the initial source (the Ca^{2+} channel), is sequestered in the ER, or is pumped out of the cell.

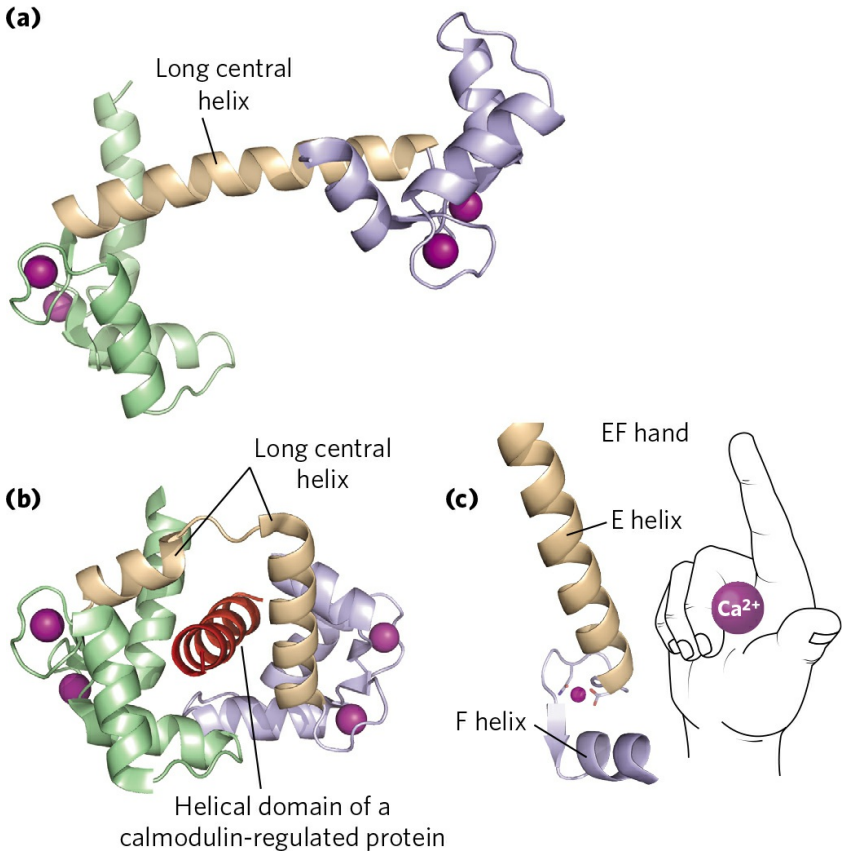


FIGURE 12-12 Calmodulin. This is the protein mediator of many Ca^{2+}-stimulated enzymatic reactions. Calmodulin has four high-affinity Ca^{2+}-binding sites ($K_d \approx 0.1$ to 1 μM). **(a)** A ribbon model of the crystal structure of calmodulin. The four Ca^{2+}-binding sites are occupied by Ca^{2+} (purple). The amino-terminal domain is on the left; the carboxyl-terminal domain on the right. **(b)** Calmodulin associated with a helical domain of one of the many enzymes it regulates, calmodulin-dependent protein kinase II. Notice that the long central α helix of calmodulin visible in (a) has bent back on itself in binding to the helical substrate domain. The central helix of calmodulin is clearly more flexible in solution than in the crystal. **(c)** Each of the four Ca^{2+}-binding sites occurs in a helix-loop-helix motif called the EF hand, also found in many other Ca^{2+}-binding proteins. [Sources: (a) PDB ID 1CLL, R. Chattopadhyaya et al., *J. Mol. Biol.* 228:1177, 1992. (b, c) PDB ID 1CDL, W. E. Meador et al., *Science* 257:1251, 1992.]

TABLE 12-5 Some Proteins Regulated by Ca^{2+} and Calmodulin

Adenylyl cyclase (brain)

Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases I to IV)
Ca^{2+}-dependent Na^{+} channel (*Paramecium*)
Ca^{2+}-release channel of sarcoplasmic reticulum
Calcineurin (phosphoprotein phosphatase 2B)
cAMP phosphodiesterase
cAMP-gated olfactory channel
cGMP-gated Na^{+}, Ca^{2+} channels (rod and cone cells)
Glutamate decarboxylase
Myosin light-chain kinases
NAD^{+} kinase
Nitric oxide synthase
Phosphatidylinositol 3-kinase
Plasma membrane Ca^{2+} ATPase (Ca^{2+} pump)
RNA helicase (p68)

There is significant cross talk between the Ca^{2+} and cAMP signaling systems. In some tissues, both the enzyme that produces cAMP (adenylyl cyclase) and the enzyme that degrades cAMP (phosphodiesterase) are stimulated by Ca^{2+}. Temporal and spatial changes in [Ca^{2+}] can therefore produce transient, localized changes in [cAMP]. We have noted already that PKA, the enzyme that responds to cAMP, is often part of a highly localized supramolecular complex assembled on scaffold proteins such as AKAPs. This subcellular localization of target enzymes, combined with temporal and spatial gradients in [Ca^{2+}] and [cAMP], allows a cell to respond to one or several signals with subtly nuanced metabolic changes, localized in space and time.

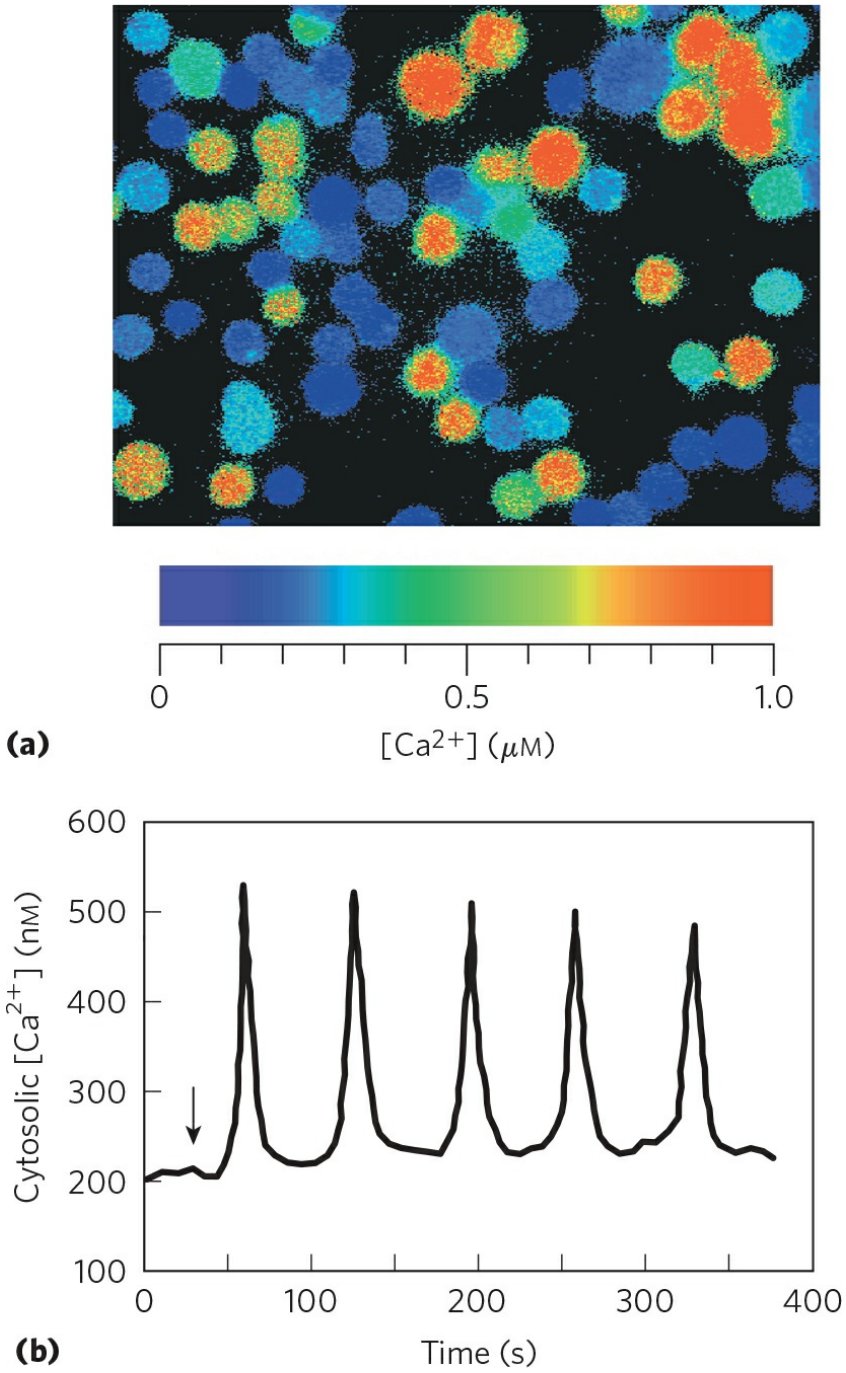


FIGURE 12-13 Triggering of oscillations in intracellular [Ca^{2+}] by extracellular signals. (a) A dye (fura) that undergoes fluorescence changes when it binds Ca^{2+} is allowed to diffuse into cells, and its instantaneous light output is measured by fluorescence microscopy. Fluorescence intensity is represented by color; the color scale relates intensity of color to [Ca^{2+}], allowing determination of the absolute [Ca^{2+}]. In this case, thymocytes (cells of the thymus) have been stimulated with extracellular ATP, which raises their internal [Ca^{2+}]. The cells are heterogeneous in their responses: some have high intracellular [Ca^{2+}] (red), others have much lower [Ca^{2+}] (blue). **(b)**

When such a probe is used in a single hepatocyte, the agonist norepinephrine (added at the arrow) causes oscillations of [Ca^{2+}] from 200 to 500 nM. Similar oscillations are induced in other cell types by other extracellular signals. [Sources: (a) Courtesy Michael D. Cahalan, Department of Physiology and Biophysics, University of California, Irvine. (b) T. A. Rooney et al., *J. Biol. Chem.* 264:17,131, 1989.]

SUMMARY 12.2 G Protein–Coupled Receptors and Second Messengers

- G protein–coupled receptors (GPCRs) share a common structural arrangement of seven transmembrane helices and act through heterotrimeric G proteins. On ligand binding, GPCRs catalyze the exchange of GTP for GDP on the G protein, causing dissociation of the G_α subunit; G_α then stimulates or inhibits the activity of an effector enzyme, changing the local concentration of its second-messenger product.
- The β-adrenergic receptor activates a stimulatory G protein, G_s, thereby activating adenylyl cyclase and raising the concentration of the second messenger cAMP. Cyclic AMP stimulates cAMP-dependent protein kinase to phosphorylate key target enzymes, changing their activities.
- Enzyme cascades, in which a single molecule of hormone activates a catalyst to activate another catalyst, and so on, result in the large signal amplification that is characteristic of hormone receptor systems.
- Cyclic AMP concentration is eventually reduced by cAMP phosphodiesterase, and G_s turns itself off by hydrolysis of its bound GTP to GDP, acting as a self-limiting binary switch.
- When the epinephrine signal persists, β-adrenergic receptor–specific protein kinase and β-arrestin temporarily desensitize the receptor and cause it to move into intracellular vesicles.
- Some receptors *stimulate* adenylyl cyclase through G_s; others *inhibit* it through G_i. Thus cellular [cAMP] reflects the integrated input of two (or more) signals.
- Noncatalytic adaptor proteins such as AKAPs hold together proteins involved in a signaling process, increasing the efficiency of their interactions and, in some cases, confining the process to a specific subcellular location.

■ Some GPCRs act via a plasma membrane phospholipase C that cleaves PIP_2 to diacylglycerol and IP_3. By opening Ca^{2+} channels in the endoplasmic reticulum, IP_3 raises cytosolic [Ca^{2+}]. Diacylglycerol and Ca^{2+} act together to activate protein kinase C, which phosphorylates and changes the activity of specific cellular proteins. Cellular [Ca^{2+}] also regulates (often through calmodulin) many other enzymes and proteins involved in secretion, cytoskeletal rearrangements, or contraction.

12.3 GPCRs in Vision, Olfaction, and Gustation

The detection of light, odors, and tastes (vision, olfaction, and gustation, respectively) in animals is accomplished by specialized sensory neurons that use signal-transduction mechanisms fundamentally similar to those that detect hormones, neurotransmitters, and growth factors. An initial sensory signal is greatly amplified by mechanisms that include gated ion channels and intracellular second messengers; the system adapts to continued stimulation by changing its sensitivity to the stimulus (desensitization); and sensory input from several receptors is integrated before the final signal goes to the brain.

The Vertebrate Eye Uses Classic GPCR Mechanisms

Visual transduction **(Fig. 12-14)** begins when light falls on **rhodopsin**, a GPCR in the disk membranes of rod cells of the vertebrate eye. (Rod cells do not detect colors; cone cells do (see below).) The light-absorbing pigment (chromophore) 11-*cis*-retinal is covalently attached to **opsin**, the protein component of rhodopsin, which lies near the middle of the disk membrane bilayer. When a photon is absorbed by the retinal component of rhodopsin (step 1), the energy causes a photochemical change; 11-*cis*-retinal is converted to all-*trans*-retinal (see Figs 1-20b and 10-20). This change in the structure of the chromophore forces conformational changes in the rhodopsin molecule, allowing it to interact with and thus activate its trimeric G protein, transducin. Rhodopsin now stimulates the exchange of bound GDP on transducin for GTP from the cytosol (Fig. 12-14, step 2), and activated transducin stimulates the membrane protein cyclic GMP (cGMP) phosphodiesterase (PDE) by removing an inhibitory subunit (step 3). The activated cGMP PDE degrades the second messenger 3′,5′-cGMP to 5′-GMP, lowering the concentration of cGMP (step 4). A cGMP-dependent Na^+ or Ca^{2+} channel in the plasma membrane closes (step 5), while a Na^+-Ca^{2+} active antiporter continues to pump Ca^{2+} outward across the plasma membrane (step 6), making the transmembrane electrical potential more negative inside (that is, hyperpolarizing the rod cell). This electrical change passes through a series of specialized nerve cells to the visual cortex of the brain.

Several steps in the visual-transduction process result in a huge amplification of the signal. Each excited rhodopsin molecule activates at least 500 molecules of transducin, and each transducin molecule can

activate a molecule of cGMP PDE. This phosphodiesterase has a remarkably high turnover number: each activated molecule hydrolyzes 4,200 molecules of cGMP per second. The binding of cGMP to cGMP-gated ion channels is cooperative, and a relatively small change in [cGMP] therefore registers as a large change in ion conductance. The result of these amplifications is exquisite sensitivity to light. Absorption of a single photon closes 1,000 or more ion channels for Na^+ and Ca^{2+}, hyperpolarizing the cell's membrane potential (V_m) by about 1 mV.

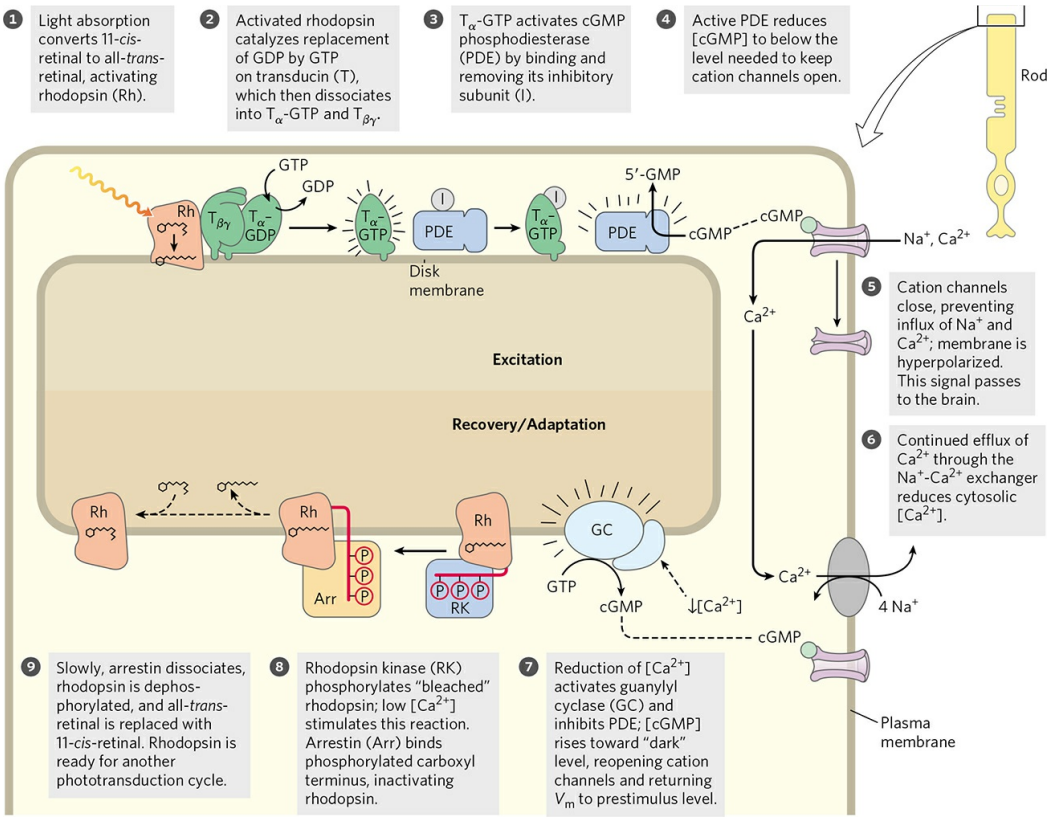


FIGURE 12-14 Molecular consequences of photon absorption by rhodopsin in the rod outer segment. The top half of the figure (steps 1 to 5) describes excitation; the bottom shows post-illumination steps: recovery (steps 6 and 7) and adaptation (steps 8 and 9).

As your eyes move across this line, the retinal images of the first words disappear rapidly—before you see the next series of words. In that short interval, a great deal of biochemistry has taken place. Very soon after illumination of the rod or cone cells stops, the photosensory system shuts off. The α subunit of transducin (T_α, with bound GTP) has intrinsic GTPase activity. Within milliseconds after the decrease in light intensity, GTP is hydrolyzed and T_α reassociates with $T_{\beta\gamma}$. The inhibitory subunit of

PDE, which had been bound to T_α-GTP, is released and reassociates with PDE, strongly inhibiting its activity and thus slowing cGMP breakdown.

At the same time, a second factor that helps to end the response to light is the reduction of intracellular [Ca^{2+}] that results from continued Ca^{2+} efflux through the Na^+-Ca^{2+} exchanger (Fig. 12-14, step 6). High [Ca^{2+}] inhibits the enzyme that makes cGMP (guanylyl cyclase; step 7), so cGMP production rises when [Ca^{2+}] falls, quickly reaching its prestimulus level.

In response to prolonged illumination, rhodopsin itself undergoes changes that limit the duration of its signaling activity. The conformational change induced in rhodopsin by light absorption exposes several Thr and Ser residues in its carboxyl-terminal domain, and these residues are phosphorylated by **rhodopsin kinase** (step 8), which is functionally and structurally homologous to the *β*-adrenergic kinase (*β*ARK) that desensitizes the *β*-adrenergic receptor. The phosphorylated carboxyl-terminal domain of rhodopsin is bound by the protein **arrestin 1**, preventing further interaction between activated rhodopsin and transducin. Arrestin 1 is a close homolog of arrestin 2 (*β*arr) of the *β*-adrenergic system. On a much longer time scale (step 9), the all-*trans*-retinal bound to light-bleached rhodopsin is removed and replaced with 11-*cis*-retinal, making rhodopsin ready to detect another photon.

Color vision involves a path of sensory transduction in cone cells essentially identical to that described for rod cells, but triggered by slightly different light receptors. Three types of cone cells are specialized to detect light from different regions of the spectrum, using three related photoreceptor proteins (opsins). Each cone cell expresses only one kind of opsin, but each type is closely related to rhodopsin in size, amino acid sequence, and, presumably, three-dimensional structure. The differences among the opsins, however, are great enough to place the chromophore, 11-*cis*-retinal, in three slightly different environments, with the result that the three photoreceptors have different absorption spectra **(Fig. 12-15)**. We discriminate colors and hues by integrating the output from the three types of cone cells, each containing one of the three types of photoreceptors.

Color blindness, such as the inability to distinguish red from green, is a fairly common, genetically inherited trait in humans. The various types of color blindness result from different opsin mutations. One form is due to loss of the red photoreceptor; affected individuals are **red$^-$ dichromats** (they see only two primary colors). Others lack the green pigment and are **green$^-$ dichromats**. In some cases, the red and green

photoreceptors are present but have a changed amino acid sequence that causes a change in their absorption spectra, resulting in abnormal color vision. Depending on which pigment is altered, these individuals are **red-anomalous trichromats** or **green-anomalous trichromats**. Examination of the genes for the visual receptors has allowed the diagnosis of color blindness in a famous "patient" more than a century after his death (Box 12-3). ■

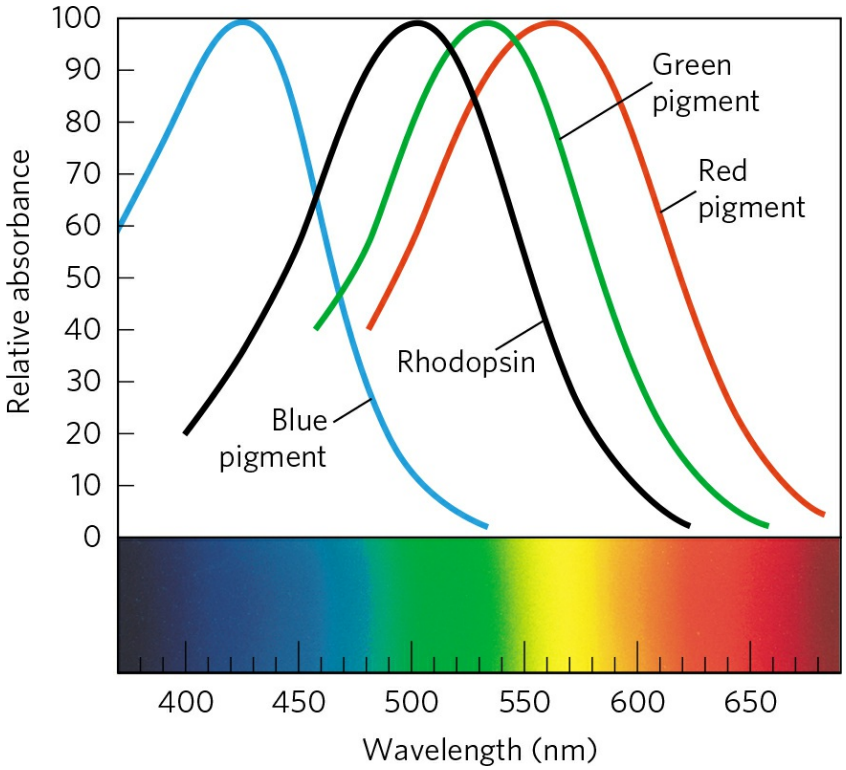


FIGURE 12-15 Absorption spectra of purified rhodopsin and the red, green, and blue receptors of cone cells. The receptor spectra, obtained from individual cone cells isolated from cadavers, peak at about 420, 530, and 560 nm, and the maximum absorption for rhodopsin is at about 500 nm. For reference, the visible spectrum for humans is about 380 to 750 nm.
[Source: Data from J. Nathans, *Sci. Am.* 260 (February):42, 1989.]

BOX 12-3 MEDICINE Color Blindness: John Dalton's Experiment from the Grave

The chemist John Dalton (of atomic theory fame) was color-blind. He thought it probable that the vitreous humor of his eyes (the fluid that fills the eyeball behind the lens) was tinted blue, unlike the colorless

fluid of normal eyes. He proposed that after his death, his eyes should be dissected and the color of the vitreous humor determined. His wish was honored. The day after Dalton's death in July 1844, Joseph Ransome dissected his eyes and found the vitreous humor to be perfectly colorless. Ransome, like many scientists, was reluctant to throw samples away. He placed Dalton's eyes in a jar of preservative, where they stayed for a century and a half (Fig. 1).

Then, in the mid-1990s, molecular biologists in England took small samples of Dalton's retinas and extracted DNA. Using the known gene sequences for the opsins of the red and green light receptors, they amplified the relevant sequences (using techniques described in Chapter 8) and determined that Dalton had the opsin gene for the red photopigment but lacked the opsin gene for the green photopigment. Dalton was a green$^-$ dichromat. So, 150 years after his death, the experiment Dalton started—by hypothesizing about the cause of his color blindness—was finally finished.

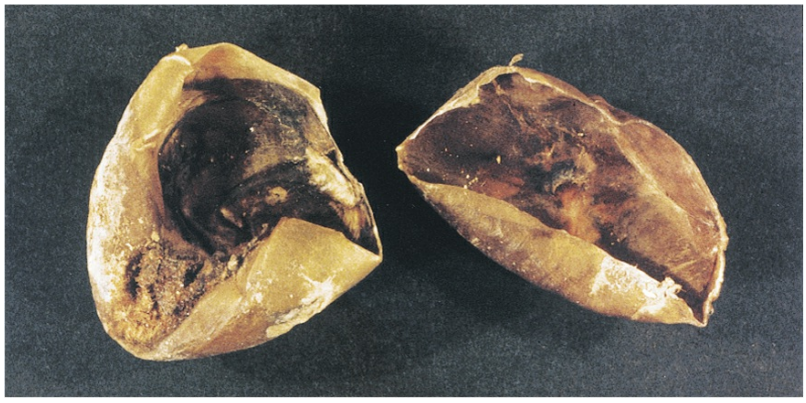


FIGURE 1 Dalton's eyes.
[Source: Professor J. D. Mollon, Department of Experimental Psychology, Cambridge University.]

Vertebrate Olfaction and Gustation Use Mechanisms Similar to the Visual System

The sensory cells that detect odors and tastes have much in common with the visual receptor system. Binding of an odorant molecule to its specific GPCR triggers a change in receptor conformation, activating a G protein, G_{olf}, analogous to transducin and to G_s of the β-adrenergic system. The activated G_{olf} activates adenylyl cyclase, raising the local [cAMP]. The cAMP-gated Na^+ and Ca^{2+} channels of the plasma membrane open, and

the influx of Na^+ and Ca^{2+} produces a small depolarization called the **receptor potential**. If a sufficient number of odorant molecules encounter receptors, the receptor potential is strong enough to cause the neuron to fire an action potential. This signal is relayed to the brain in several stages and registers as a specific smell. All these events occur within 100 to 200 ms. When the olfactory stimulus is no longer present, the transducing machinery shuts itself off in several ways. A cAMP phosphodiesterase returns [cAMP] to the prestimulus level. G_{olf} hydrolyzes its bound GTP to GDP, thereby inactivating itself. Phosphorylation of the receptor by a specific kinase prevents its interaction with G_{olf}, by a mechanism analogous to that used to desensitize the β-adrenergic receptor and rhodopsin. Some odorants are detected by another mechanism we have seen in other signal transductions: activation of a phospholipase and production of IP_3, leading to a rise in intracellular $[Ca^{2+}]$.

The sense of taste in vertebrates reflects the activity of gustatory neurons clustered in taste buds on the surface of the tongue. For example, sweet-tasting molecules are those that bind receptors in “sweet” taste buds. In taste sensory neurons, GPCRs are coupled to the heterotrimeric G protein **gustducin**. When the tastant molecule binds its receptor, gustducin is activated and stimulates cAMP production by adenylyl cyclase. The resulting elevation of [cAMP] activates PKA, which phosphorylates K^+ channels in the plasma membrane, causing them to close and sending an electrical signal to the brain. Other taste buds specialize in detecting bitter, sour, salty, or umami (savory) tastants, using various combinations of second messengers and ion channels in the transduction mechanisms.

All GPCR Systems Share Universal Features

We have now looked at several types of signaling systems (hormone signaling, vision, olfaction, and gustation) in which membrane receptors are coupled to second messenger–generating enzymes through G proteins. As we have intimated, signaling mechanisms must have arisen early in evolution; genomic studies have revealed hundreds of genes encoding GPCRs in vertebrates, arthropods (*Drosophila* and mosquito), and the roundworm *Caenorhabditis elegans*. Even the common budding yeast *Saccharomyces* uses GPCRs and G proteins to detect the opposite mating type. Overall patterns have been conserved, and the introduction of variety has given modern organisms the ability to respond to a wide range of stimuli (Table 12-6). Of the approximately 20,000 genes in the human

genome, as many as 1,000 encode GPCRs, including hundreds for olfactory stimuli and many orphan receptors, for which the natural ligand is not yet known.

TABLE 12-6 Some Signals That Act through GPCRs

Amines
Acetylcholine (muscarinic)
Dopamine
Epinephrine
Histamine
Serotonin
Peptides
Angiotensin
Bombesin
Bradykinin
Chemokine
Colecystokinin (CCK)
Endothelin
Gonadotropin-releasing hormone
Interleukin-8
Melanocortin
Neuropeptide Y
Neurotensin
Orexin
Somatostatin
Tachykinin
Thyrotropin-releasing hormone
Urotensin II
Protein hormones
Follicle-stimulating hormone
Gonadotropin

Lutropin- choriogonadotropic hormone
Thyrotropin
Prostanoids
Prostacyclin
Prostaglandin
Thromboxane
Others
Cannabinoids
Lysosphingolipids
Melatonin
Olfactory stimuli
Opioids
Rhodopsin

All well-studied signal-transducing systems that act through heterotrimeric G proteins share some common features that reflect their evolutionary relatedness **(Fig. 12-16)**. The receptors have seven transmembrane segments, a domain (generally the loop between transmembrane helices 6 and 7) that interacts with a G protein, and a carboxyl-terminal cytoplasmic domain that undergoes reversible phosphorylation on several Ser or Thr residues. The ligand-binding site (or, in the case of light reception, the light receptor) is buried deep in the membrane and includes residues from several of the transmembrane segments. Ligand binding (or light) induces a conformational change in the receptor, exposing a domain that can interact with a G protein. Heterotrimeric G proteins activate or inhibit effector enzymes (adenylyl cyclase, PDE, or PLC), which change the concentration of a second messenger (cAMP, cGMP, IP_3, or Ca^{2+}). In the hormone-detecting systems, the final output is an activated protein kinase that regulates some cellular process by phosphorylating a protein critical to that process. In sensory neurons, the output is a change in membrane potential and a consequent electrical signal that passes to another neuron in the pathway connecting the sensory cell to the brain.

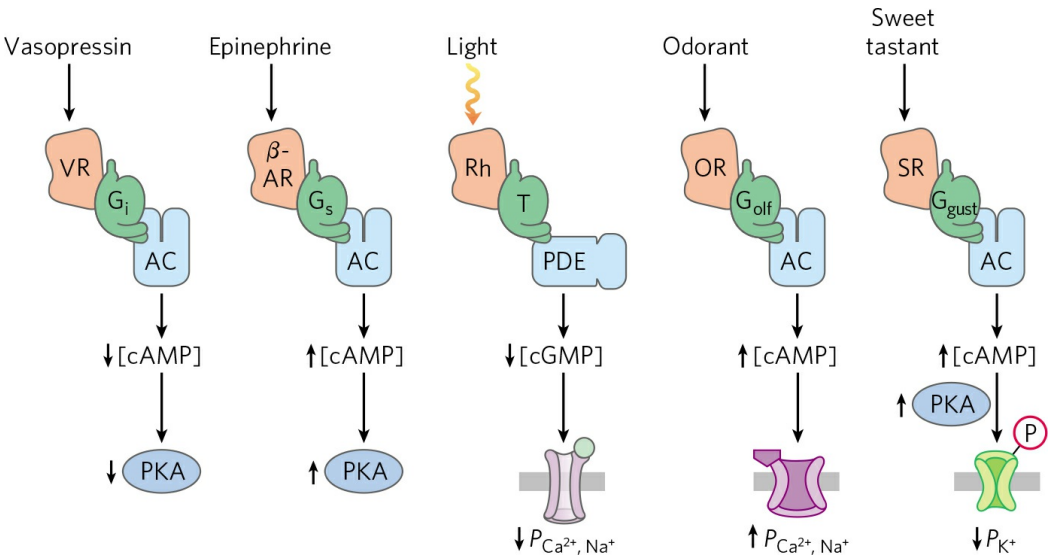


FIGURE 12-16 Common features of signaling systems that detect hormones, light, smells, and tastes. GPCRs provide signal specificity, and their interaction with G proteins provides signal amplification. Heterotrimeric G proteins activate effector enzymes: adenylyl cyclase (AC) and phosphodiesterases (PDEs) that degrade cAMP or cGMP. Changes in concentration of the second messengers (cAMP, cGMP) result in alterations in enzymatic activities via phosphorylation or alterations in the permeability (*P*) of surface membranes to Ca^{2+}, Na^+, and K^+. The resulting depolarization or hyperpolarization of the sensory cell (the signal) passes through relay neurons to sensory centers in the brain. In the best-studied cases, desensitization includes phosphorylation of the receptor and binding of a protein (arrestin) that interrupts receptor–G protein interactions. (The path of odorant detection by production of IP_3 and increase in intracellular $[Ca^{2+}]$, mentioned in the text, is not shown here.) VR is the vasopressin receptor; β-AR, the β-adrenergic receptor; Rh, rhodopsin; OR, olfactory receptor; SR, sweet-taste receptor.

All these systems self-inactivate. Bound GTP is converted to GDP by the intrinsic GTPase activity of G proteins, often augmented by GTPase-activating proteins (GAPs) or RGS proteins (regulators of G-protein signaling; see Fig. 12-5 and Box 12-1, Fig. 4). In some cases, the effector enzymes that are the targets of modulation by G proteins also serve as GAPs. The desensitization mechanism involving phosphorylation of the carboxyl-terminal region followed by arrestin binding is widespread and may be universal.

Each of the 1,000 GPCRs of vertebrates is expressed selectively, in certain cell types or under certain conditions. Together, they allow cells and tissues to respond to a wide array of stimuli, including various low

molecular weight amines, peptides, proteins, eicosanoids and other lipids, as well as light and the many compounds detected by olfaction and gustation. The determination of several GPCR structures by crystallography **(Fig. 12-17)**, including the β-adrenergic receptor and the histamine receptor, has stimulated great interest in both the transduction mechanism(s) and the possibilities of altering receptor activity with drugs. These two receptors are the targets of a variety of widely used beta-blocker and antihistamine medications, respectively. The structural similarities among GPCRs go beyond the common seven–transmembrane helix pattern; as Figure 12-17d shows, the structures of five different GPCRs are almost superimposable. Clearly, something about this three-dimensional structure makes it effective as a transducer of many disparate signals.

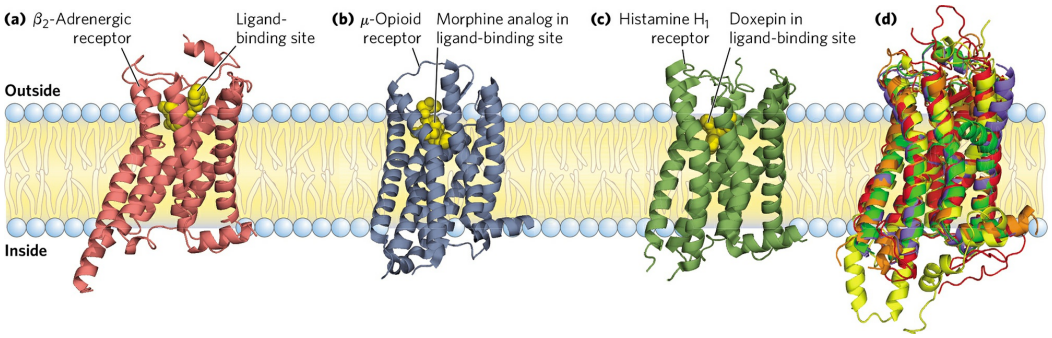


FIGURE 12-17 The β-adrenergic receptor and several other GPCRs. (a) The β_2-adrenergic receptor with the agonist epinephrine, shown in yellow, in the ligand-binding site. **(b)** The μ opioid receptor, the target of morphine and codeine, with a morphine analog in the ligand-binding site. **(c)** The histamine H_1 receptor with the bound drug doxepin. **(d)** Five GPCR structures superimposed to show the remarkable conservation of structure. Shown are the human A2A adenosine receptor (orange); turkey β_1-adrenergic receptor (blue), human β_2-adrenergic receptor (green), squid rhodopsin (yellow); and bovine rhodopsin (red). [Sources: (a) PDB ID 3SN6, S. G. F. Rasmussen et al., *Nature* 477:549, 2011. (b) PDB ID 4DKL, A. Manglik et al., *Nature* 485:321, 2012. (c) PDB ID 3RZE, T. Shimamura et al., *Nature* 475:65, 2011. (d) Human A2A adenosine receptor: PDB ID 3EML, V. P. Jaakola et al., *Science* 322:1211, 2008; turkey β_1-adrenergic receptor: PDB ID 2VT4, A. Warne et al., *Nature* 454:486, 2008; human β_2-adrenergic receptor: PDB ID 2RH1, V. Cherezov et al., *Science* 318:1258, 2007; squid rhodopsin: PDB ID 2Z73, M. Murakami and T. Kouyama, *Nature* 453:363, 2008; bovine rhodopsin: PDB ID 1U19, T. Okada et al., *J. Mol. Biol.* 342:571, 2004.]

SUMMARY 12.3 GPCRs in Vision, Olfaction, and Gustation

- Vision, olfaction, and gustation in vertebrates employ GPCRs, which act through heterotrimeric G proteins to change the membrane potential (V_m) of a sensory neuron.
- In rod and cone cells of the retina, light activates rhodopsin, which activates the G protein transducin. The freed α subunit of transducin activates a cGMP phosphodiesterase, which lowers [cGMP] and thus closes cGMP-dependent ion channels in the outer segment of the neuron. The resulting hyperpolarization of the rod or cone cell carries the signal to the next neuron in the pathway, and eventually to the brain.
- In olfactory neurons, olfactory stimuli, acting through GPCRs and G proteins, trigger an increase in [cAMP] (by activating adenylyl cyclase) or [Ca^{2+}] (by activating PLC). These second messengers affect ion channels and thus the V_m.
- Gustatory neurons have GPCRs that respond to tastants by altering levels of cAMP, which changes V_m by gating ion channels.
- There is a high degree of conservation of signaling proteins and transduction mechanisms across signaling systems and across species.

12.4 Receptor Tyrosine Kinases

The **receptor tyrosine kinases (RTKs)**, a family of plasma membrane receptors with intrinsic protein kinase activity, transduce extracellular signals by a mechanism fundamentally different from that of GPCRs. RTKs have a ligand-binding domain on the extracellular face of the plasma membrane and an enzyme active site on the cytoplasmic face, connected by a single transmembrane segment. The cytoplasmic domain is a protein kinase that phosphorylates Tyr residues (a Tyr kinase) in specific target proteins. The receptors for insulin and epidermal growth factor are prototypes for the approximately 60 RTKs in humans.

Stimulation of the Insulin Receptor Initiates a Cascade of Protein Phosphorylation Reactions

Insulin regulates both metabolic enzymes and gene expression. Insulin does not enter cells, but initiates a signal that travels a branched pathway from the plasma membrane receptor to insulin-sensitive enzymes in the cytosol, and to the nucleus, where it stimulates the transcription of specific genes. The active insulin receptor protein (INSR) consists of two identical α subunits protruding from the outer face of the plasma membrane and two transmembrane β subunits with their carboxyl termini protruding into the cytosol—a dimer of $\alpha\beta$ monomers **(Fig. 12-18)**. The α subunits contain the insulin-binding domain, and the intracellular domains of the β subunits contain the protein kinase activity that transfers a phosphoryl group from ATP to the hydroxyl group of Tyr residues in specific target proteins. Signaling through INSR begins when the binding of one insulin molecule between the two subunits of the dimer activates the Tyr kinase activity, and each β subunit phosphorylates three critical Tyr residues near the carboxyl terminus of the other β subunit. This **autophosphorylation** opens the active site so that the enzyme can phosphorylate Tyr residues of other target proteins. The mechanism of activation of the INSR protein kinase is similar to that described for PKA and PKC: a region of the cytoplasmic domain (an autoinhibitory sequence) that usually occludes the active site moves out of the active site after being phosphorylated, opening the site for the binding of target proteins (Fig. 12-18).

When INSR is autophosphorylated (**Fig. 12-19**, step 1) and becomes an active Tyr kinase, one of its targets is insulin receptor substrate-1 (IRS-1; step 2). Once phosphorylated on several of its Tyr residues, IRS-1 becomes the point of nucleation for a complex of proteins (step 3) that

carry the message from the insulin receptor to end targets in the cytosol and nucleus, through a long series of intermediate proteins. First, a Ⓟ–Tyr residue of IRS-1 binds to the **SH2 domain** of the protein Grb2. (SH2 is an abbreviation of *S*rc *h*omology *2*, so named because the sequence of an SH2 domain is similar to that of a domain in Src (pronounced *sark*), another protein Tyr kinase.) Many signaling proteins contain SH2 domains, all of which bind Ⓟ–Tyr residues in a protein partner. Grb2 (*g*rowth factor *r*eceptor-*b*ound protein *2*) is an adaptor protein, with no intrinsic enzymatic activity. Its function is to bring together two proteins (in this case, IRS-1 and the protein Sos) that must interact to enable signal transduction. In addition to its SH2 (Ⓟ–Tyr-binding) domain, Grb2 contains a second protein-binding domain, SH3, that binds to a proline-rich region of Sos, recruiting Sos to the growing receptor complex. When bound to Grb2, Sos acts as a guanosine nucleotide–exchange factor (GEF), catalyzing the replacement of bound GDP with GTP on Ras, a G protein.

Ras is the prototype of a family of **small G proteins** that mediate a wide variety of signal transductions (see Box 12-1). Like the trimeric G protein that functions with the β-adrenergic system (Fig. 12-5), Ras can exist in either the GTP-bound (active) or GDP-bound (inactive) conformation, but Ras (~20 kDa) acts as a monomer. When GTP binds, Ras can activate a protein kinase, Raf-1 (Fig. 12-19, step ❹), the first of three protein kinases—Raf-1, MEK, and ERK—that form a cascade in which each kinase activates the next by phosphorylation (step ❺). The protein kinases MEK and ERK are activated by phosphorylation of both a Thr and a Tyr residue. When activated, ERK mediates some of the biological effects of insulin by entering the nucleus and phosphorylating transcription factors, including Elk1 (step ❻), that modulate the transcription of about 100 insulin-regulated genes (step ❼), some of which encode proteins essential for cell division. Thus, insulin acts as a growth factor.

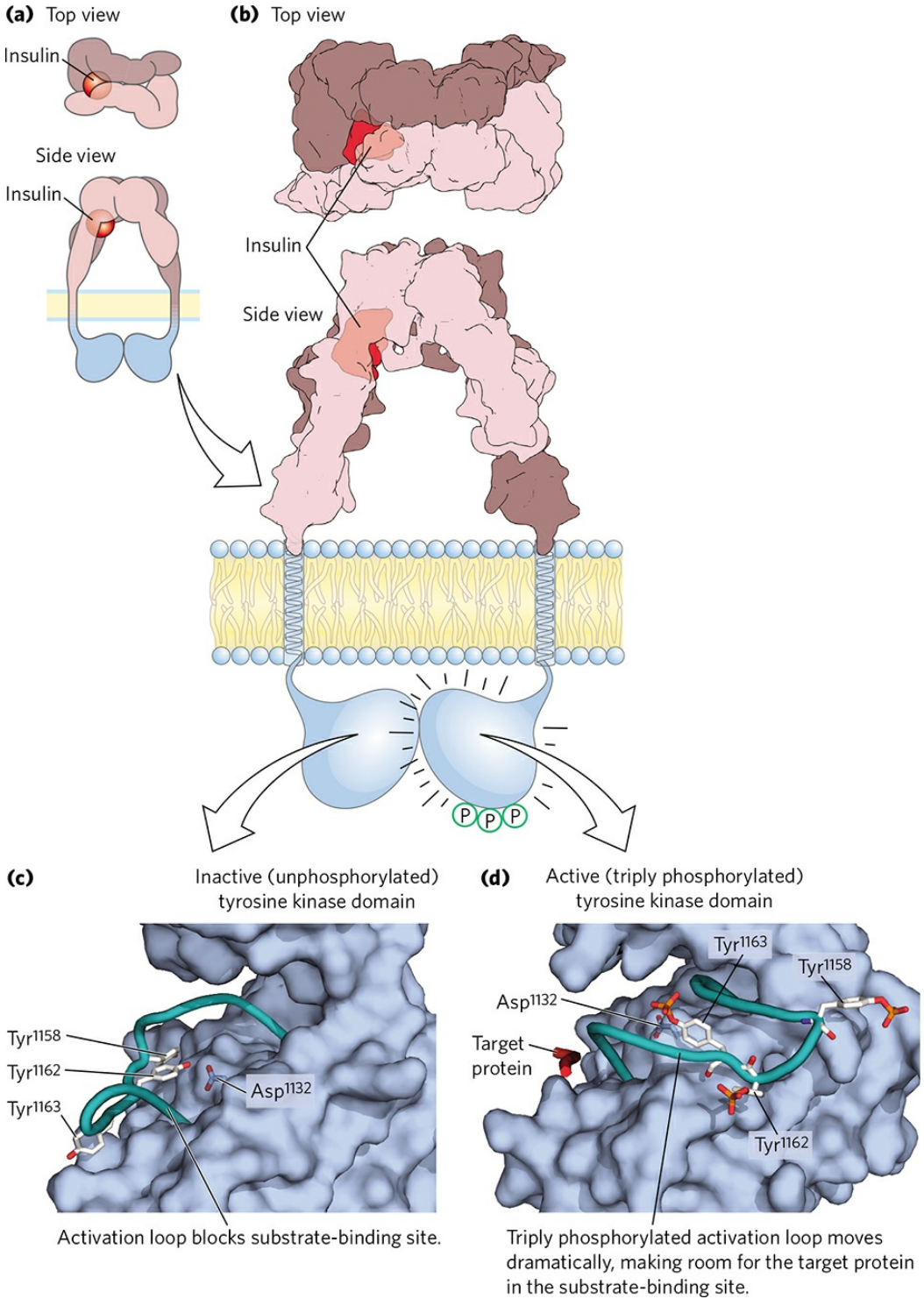


FIGURE 12-18 Activation of the insulin-receptor tyrosine kinase by autophosphorylation. (a) The insulin-binding region of the insulin receptor lies outside the cell and comprises **(b)** two α subunits and the extracellular portions of two β subunits, intertwined to form the insulin-binding site (shown as a surface contour model of the crystal structure). (The structure of the transmembrane domain has not been solved by crystallography.) The binding of insulin is communicated through the single transmembrane helix of each β subunit to the paired Tyr kinase

domains inside the cell, activating them to phosphorylate each other on three Tyr residues. **(c)** In the inactive form of the Tyr kinase domain, the activation loop (backbone shown in teal) sits in the active site, and none of the critical Tyr residues (stick structures) are phosphorylated. This conformation is stabilized by hydrogen bonding between Tyr^{1162} and Asp^{1132}. **(d)** Activation of the Tyr kinase allows each β subunit to phosphorylate three Tyr residues (Tyr^{1158}, Tyr^{1162}, Tyr^{1163}) on the other β subunit. (Phosphoryl groups are depicted in red and orange.) The introduction of three highly charged Ⓟ-Tyr residues forces a 30 Å change in the position of the activation loop, away from the substrate-binding site, which thus becomes available to bind and phosphorylate a target protein. [Sources: (b) Insulin receptor: derived from PDB ID 2DTG, N. M. McKern et al., *Nature* 443:218, 2006; insulin: PDB ID 2CEU, J. L. Whittingham et al., *Acta Crystallogr. D Biol. Crystallogr.* 62:505, 2006. (c) PDB ID 1IRK, S. R. Hubbard et al., *Nature* 372:746, 1994. (d) PDB ID 1IR3, S. R. Hubbard, *EMBO J.* 16:5572, 1997.]

The proteins Raf-1, MEK, and ERK are members of three larger families, for which several nomenclatures are used. ERK is in the **MAPK** family (*m*itogen-*a*ctivated *p*rotein *k*inases; mitogens are extracellular signals that induce mitosis and cell division). Soon after discovery of the first MAPK enzyme, that enzyme was found to be activated by another protein kinase, which was named MAP kinase kinase (MEK belongs to this family), and when a third kinase that activated MAP kinase kinase was discovered, it was given the slightly ludicrous family name MAP kinase kinase kinase (Raf-1 is in this family). Somewhat less cumbersome are the abbreviations for these three families: MAPK, MAPKK, and MAPKKK. Kinases in the MAPK and MAPKKK families are specific for Ser or Thr residues, and MAPKKs (here, MEK) phosphorylate both a Ser and a Tyr residue in their substrate, a MAPK (here, ERK).

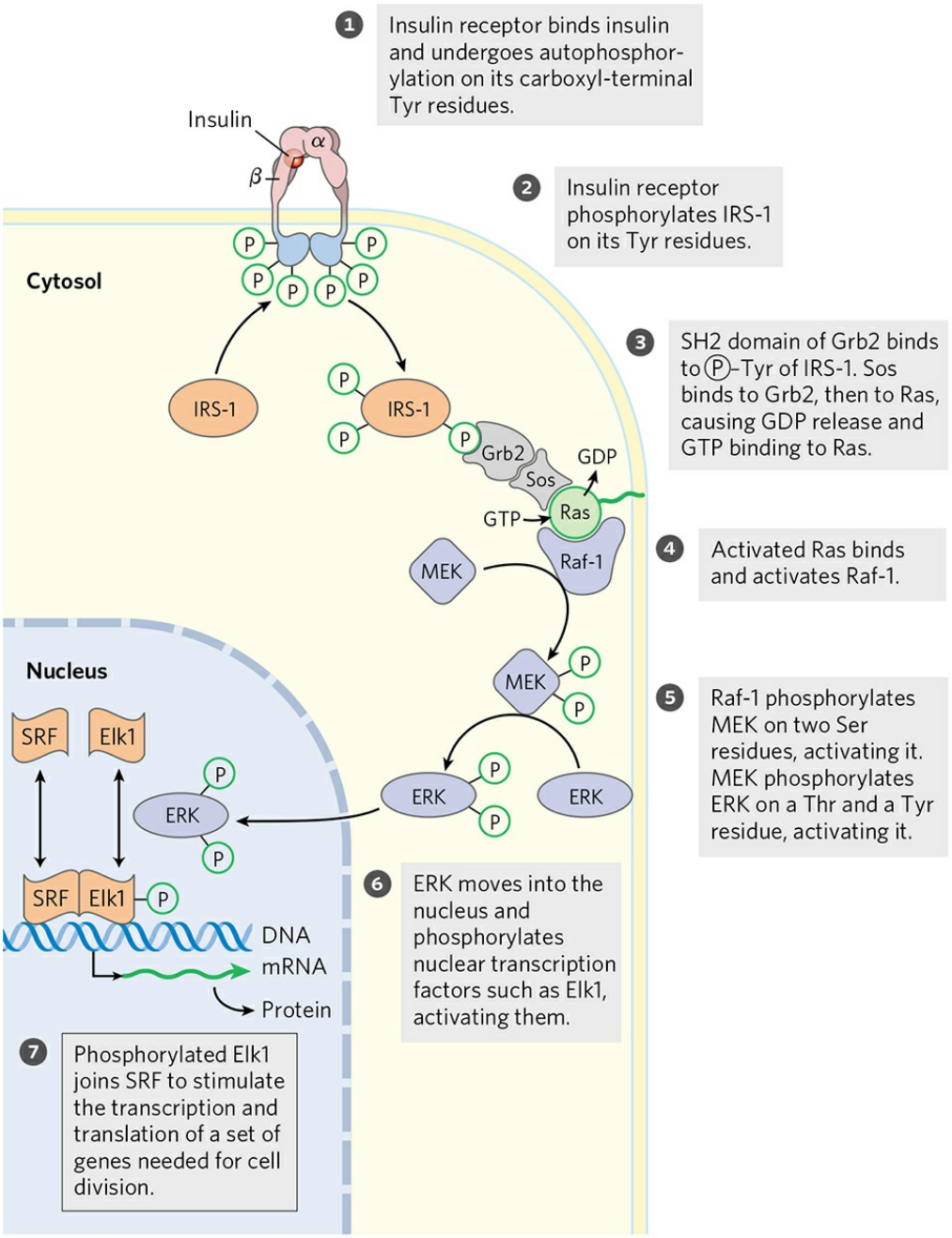


FIGURE 12-19 Regulation of gene expression by insulin through a MAP kinase cascade. The insulin receptor (INSR) consists of two α subunits on the outer face of the plasma membrane and two β subunits that traverse the membrane and protrude from the cytoplasmic face. Binding of insulin to the α subunits triggers a conformational change that allows the autophosphorylation of Tyr residues in the carboxyl-terminal domain of the β subunits. Autophosphorylation further activates the Tyr kinase domain, which then catalyzes phosphorylation of other target proteins. The signaling pathway by which insulin regulates the expression of specific genes consists of a cascade of protein kinases, each of which activates the next. INSR is a Tyr-specific

kinase; the other kinases (all shown in blue) phosphorylate Ser or Thr residues. MEK is a dual-specificity kinase that phosphorylates both a Thr and a Tyr residue in ERK (*e*xtracellular *r*egulated *k*inase). MEK is *m*itogen-activated, *E*RK-activating *k*inase; SRF is *s*erum *r*esponse *f*actor.

Biochemists now recognize this insulin pathway as but one instance of a more general scheme in which hormone signals, via pathways similar to that shown in Figure 12-19, result in a change in the phosphorylation of target enzymes by protein kinases or phosphoprotein phosphatases. The target of phosphorylation is often another protein kinase, which then phosphorylates a third protein kinase, and so on. The result is a cascade of reactions that amplifies the initial signal by many orders of magnitude (see Fig. 12-1b). **MAPK cascades** (Fig. 12-19) mediate signaling initiated by a variety of growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Another general scheme exemplified by the insulin receptor pathway is the use of nonenzymatic adaptor proteins to bring together the components of a branched signaling pathway, to which we now turn.

The Membrane Phospholipid PIP_3 Functions at a Branch in Insulin Signaling

The signaling pathway from insulin branches at IRS-1 (Fig. 12-19, step ❷). Grb2 is not the only protein that associates with phosphorylated IRS-1. The enzyme phosphoinositide 3-kinase (PI3K) binds IRS-1 through PI3K's SH2 domain **(Fig. 12-20)**. Thus activated, PI3K converts the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) by the transfer of a phosphoryl group from ATP. The multiply (negatively) charged head group of PIP_3, protruding on the cytoplasmic side of the plasma membrane, is the starting point for a second signaling branch involving another cascade of protein kinases. When bound to PIP_3, protein kinase B (PKB; also called Akt) is phosphorylated and activated by yet another protein kinase, PDK1. The activated PKB then phosphorylates Ser or Thr residues in its target proteins, one of which is glycogen synthase kinase 3 (GSK3). In its active, nonphosphorylated form, GSK3 phosphorylates glycogen synthase, inactivating it and thereby contributing to the slowing of glycogen synthesis. (This mechanism is only part of the explanation for the effects of insulin on glycogen metabolism; see Fig. 15-42.) When

phosphorylated by PKB, GSK3 is inactivated. By thus preventing inactivation of glycogen synthase in liver and muscle, the cascade of protein phosphorylations initiated by insulin stimulates glycogen synthesis (Fig. 12-20). In a third signaling branch in muscle and fat tissue, PKB triggers the clathrin-aided movement of glucose transporters (GLUT4) from intracellular vesicles to the plasma membrane, stimulating glucose uptake from the blood (Fig. 12-20, step 5; see also Box 11-1).

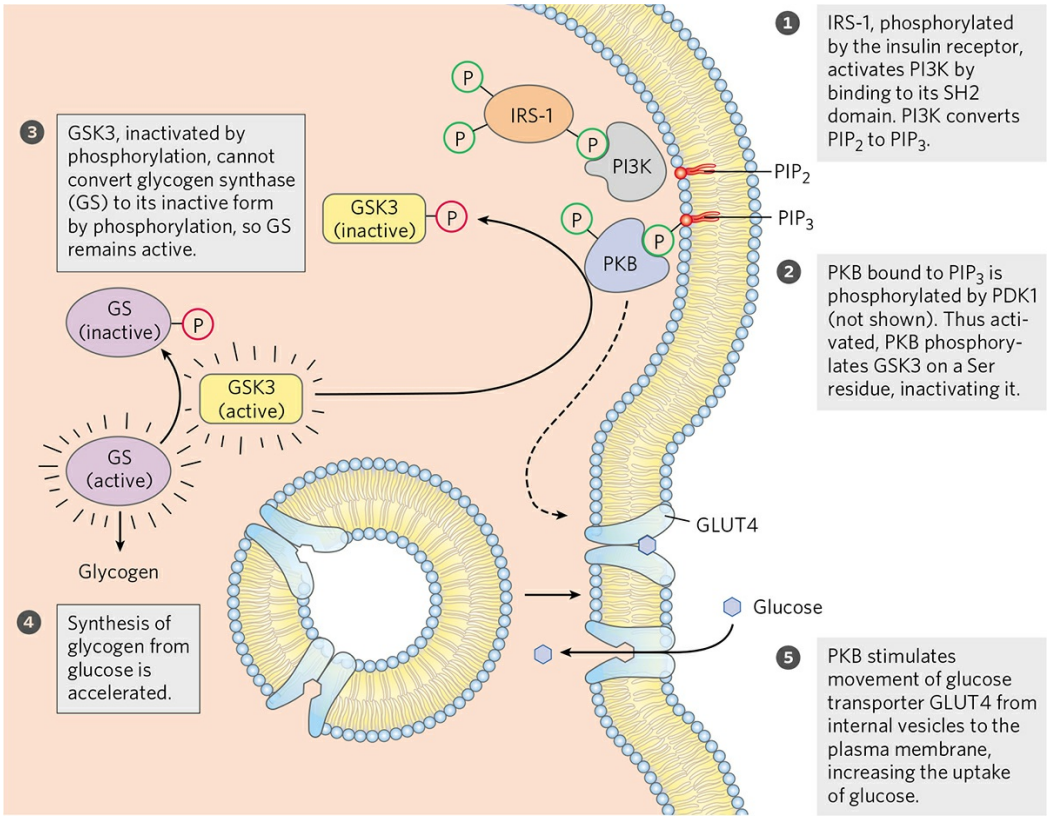


FIGURE 12-20 Insulin action on glycogen synthesis and GLUT4 movement to the plasma membrane. The activation of PI3 kinase (PI3K) by phosphorylated IRS-1 initiates (through protein kinase B, PKB) movement of the glucose transporter GLUT4 to the plasma membrane, and the activation of glycogen synthase.

As in all signaling pathways, there is a mechanism for terminating the activity of the PI3K-PKB pathway. A PIP_3-specific phosphatase (PTEN in humans) removes the phosphoryl group at the 3 position of PIP_3 to produce PIP_2, which no longer serves as a binding site for PKB, and the signaling chain is broken. In various types of cancer, it is often found that the PTEN gene has undergone mutation, resulting in a defective regulatory circuit and abnormally high levels of PIP_3 and of PKB activity. The result is a continuing signal for cell division and thus tumor growth. ■

The insulin receptor is the prototype for several receptor enzymes with a similar structure and RTK activity **(Fig. 12-21)**. The receptors for EGF and PDGF, for example, have structural and sequence similarities to INSR, and both have a protein Tyr kinase activity that phosphorylates IRS-1. Many of these receptors dimerize after binding ligand; INSR is the exception, as it is already an $(\alpha\beta)_2$ dimer before insulin binds. (The protomer of the insulin receptor is one $\alpha\beta$ unit.) The binding of adaptor proteins such as Grb2 to Ⓟ–Tyr residues is a common mechanism for promoting protein-protein interactions initiated by RTKs, a subject to which we return in Section 12.6.

In addition to the many receptors that act as protein Tyr kinases (the RTKs), several receptorlike plasma membrane proteins have protein Tyr phosphatase activity. Based on the structures of these proteins, we can surmise that their ligands are components of the extracellular matrix or are surface molecules on other cells. Although their signaling roles are not yet as well understood as those of the RTKs, they clearly have the potential to reverse the actions of signals that stimulate RTKs.

What spurred the evolution of such complicated regulatory machinery? This system allows one activated receptor to activate several IRS-1 molecules, amplifying the insulin signal, and it provides for the integration of signals from different receptors such as EGFR and PDGFR, each of which can phosphorylate IRS-1. Furthermore, because IRS-1 can activate any of several proteins that contain SH2 domains, a single receptor acting through IRS-1 can trigger two or more signaling pathways; insulin affects gene expression through the Grb2-Sos-Ras-MAPK pathway and affects glycogen metabolism and glucose transport through the PI3K-PKB pathway. Finally, there are several closely related IRS proteins (IRS2, IRS3), each with its own characteristic tissue distribution and function, further enriching the signaling possibilities in pathways initiated by RTKs.

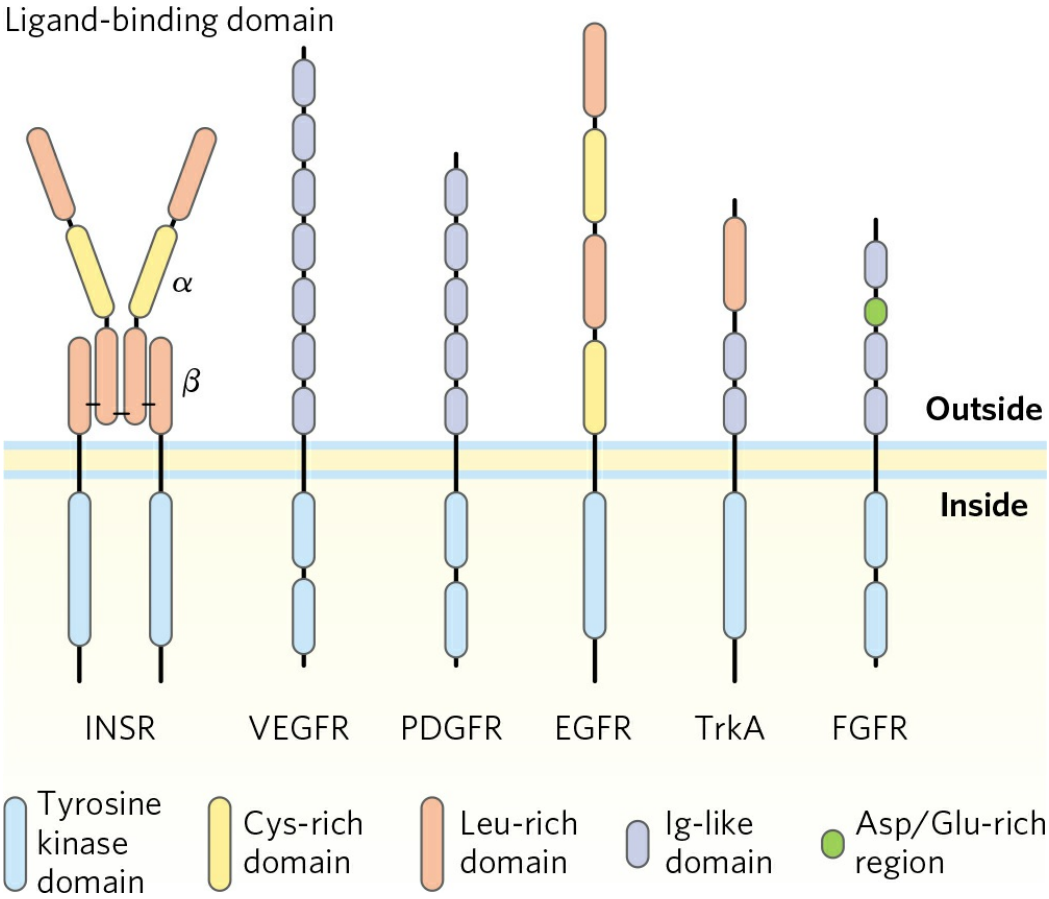


FIGURE 12-21 Receptor tyrosine kinases. Growth factor receptors that initiate signals through Tyr kinase activity include those for insulin (INSR), vascular epidermal growth factor (VEGFR), platelet-derived growth factor (PDGFR), epidermal growth factor (EGFR), high-affinity nerve growth factor (TrkA), and fibroblast growth factor (FGFR). All these receptors have a Tyr kinase domain on the cytoplasmic side of the plasma membrane (blue). The extracellular domain is unique to each type of receptor, reflecting the different growth-factor specificities. These extracellular domains are typically combinations of structural motifs such as cysteine- or leucine-rich segments and segments containing one of several motifs common to immunoglobulins (Ig). Many other receptors of this type are encoded in the human genome, each with a different extracellular domain and ligand specificity.

Cross Talk among Signaling Systems Is Common and Complex

For simplicity, we have treated individual signaling pathways as separate sequences of events leading to separate metabolic consequences, but there is, in fact, extensive cross talk among signaling systems. The regulatory circuitry that governs metabolism is richly interwoven and multilayered. We have discussed the signaling pathways for insulin and epinephrine

separately, but they do not operate independently. Insulin opposes the metabolic effects of epinephrine in most tissues, and activation of the insulin signaling pathway directly attenuates signaling through the β-adrenergic signaling system. For example, the INSR kinase directly phosphorylates two Tyr residues in the cytoplasmic tail of a β_2-adrenergic receptor, and PKB, activated by insulin **(Fig. 12-22)**, phosphorylates two Ser residues in the same region. Phosphorylation of these four residues triggers clathrin-aided internalization of the β_2-adrenergic receptor, taking it out of service and lowering the cell's sensitivity to epinephrine. A second type of cross talk between these receptors occurs when Ⓟ–Tyr residues on the β_2-adrenergic receptor, phosphorylated by INSR, serve as nucleation points for SH2 domain–containing proteins such as Grb2 (Fig. 12-22, left side). Activation of the MAPK ERK by insulin (Fig. 12-19) is 5- to 10-fold greater in the presence of the β_2-adrenergic receptor, presumably because of this cross talk. Signaling systems that use cAMP and Ca^{2+} also show extensive interaction; each second messenger affects the generation and concentration of the other. One of the major challenges of systems biology is to sort out the effects of such interactions on the overall metabolic patterns in each tissue—a daunting task.

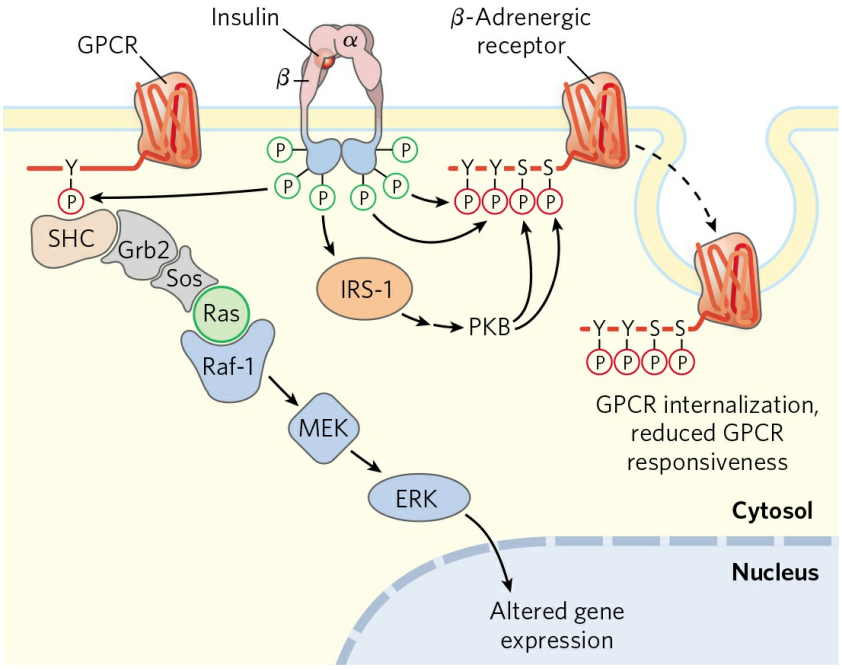


FIGURE 12-22 Cross talk between the insulin receptor and the β_2-adrenergic receptor (or other GPCR). When INSR is activated by insulin binding, its Tyr kinase directly phosphorylates the β_2-adrenergic receptor (right side) on two Tyr residues (Tyr^{350} and Tyr^{364}) near its

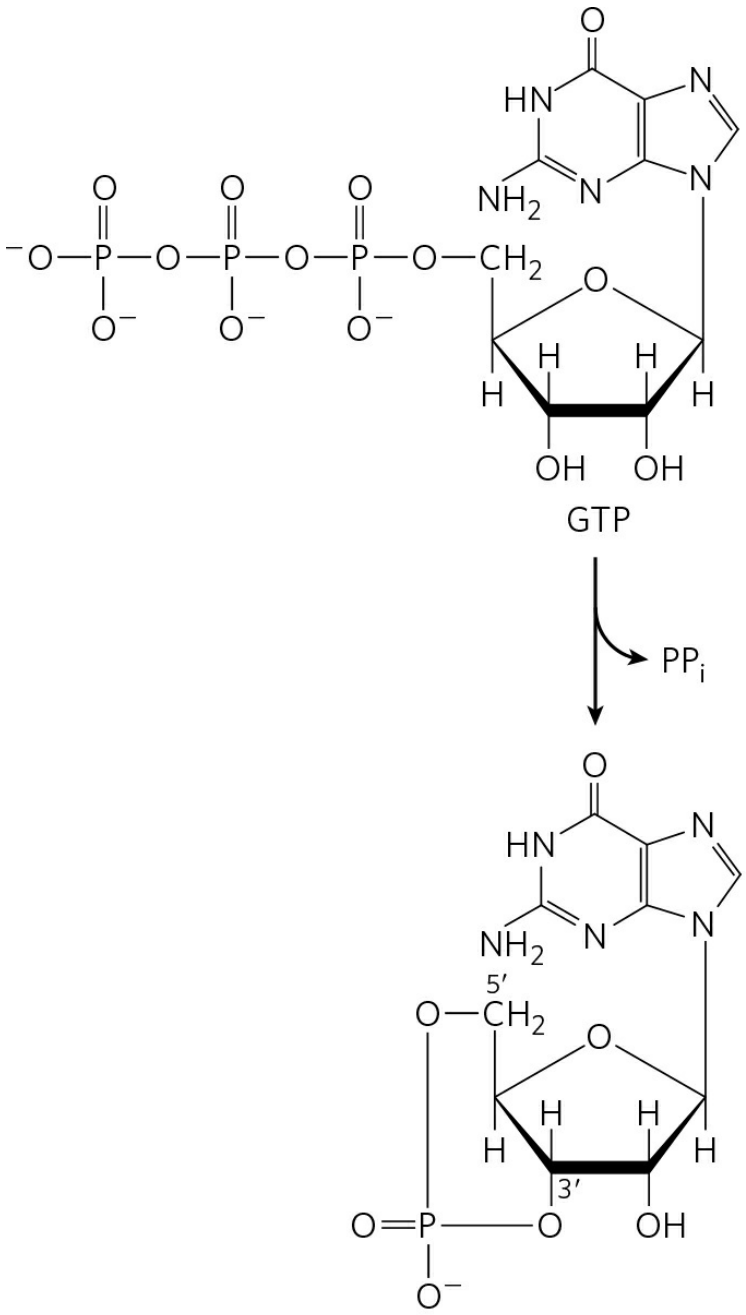
carboxyl terminus, and indirectly (through activation of protein kinase B (PKB); see Fig. 12-20) causes phosphorylation of two Ser residues in the same region. The effect of these phosphorylations is internalization of the adrenergic receptor, reducing the response to the adrenergic stimulus. Alternatively (left side), INSR-catalyzed phosphorylation of a GPCR (an adrenergic or other receptor) on a carboxyl-terminal Tyr creates the point of nucleation for activating the MAPK cascade (see Fig. 12-19), with Grb2 serving as the adaptor protein. In this case, INSR has used the GPCR to enhance its own signaling.

SUMMARY 12.4 Receptor Tyrosine Kinases

- The insulin receptor, INSR, is the prototype of receptor enzymes with Tyr kinase activity. When insulin binds, each $\alpha\beta$ unit of INSR phosphorylates the β subunit of its partner, activating the receptor's Tyr kinase activity. The kinase catalyzes the phosphorylation of Tyr residues on other proteins, such as IRS-1.
- Phosphotyrosine residues in IRS-1 serve as binding sites for proteins with SH2 domains. Some of these proteins, such as Grb2, have two or more protein-binding domains and can serve as adaptors that bring two proteins into proximity.
- Sos bound to Grb2 catalyzes GDP-GTP exchange on Ras (a small G protein), which in turn activates a MAPK cascade that ends with the phosphorylation of target proteins in the cytosol and nucleus. The result is specific metabolic changes and altered gene expression.
- The enzyme PI3K, activated by interaction with IRS-1, converts the membrane lipid PIP_2 to PIP_3, which becomes the point of nucleation for proteins in a second and third branch of insulin signaling.
- There are extensive interconnections among signaling pathways, allowing integration and fine-tuning of multiple hormonal effects.

12.5 Receptor Guanylyl Cyclases, cGMP, and Protein Kinase G

Guanylyl cyclases **(Fig. 12-23)** are receptor enzymes that, when activated, convert GTP to the second messenger 3′,5′-cyclic GMP (cGMP):



Many of the actions of cGMP in animals are mediated by **cGMP-dependent protein kinase**, also called **protein kinase G (PKG)**. On

activation by cGMP, PKG phosphorylates Ser and Thr residues in target proteins. The catalytic and regulatory domains of this enzyme are in a single polypeptide (M_r ~80,000). Part of the regulatory domain fits snugly in the substrate-binding cleft. Binding of cGMP forces this pseudosubstrate out of the binding site, opening the site to target proteins containing the PKG consensus sequence.

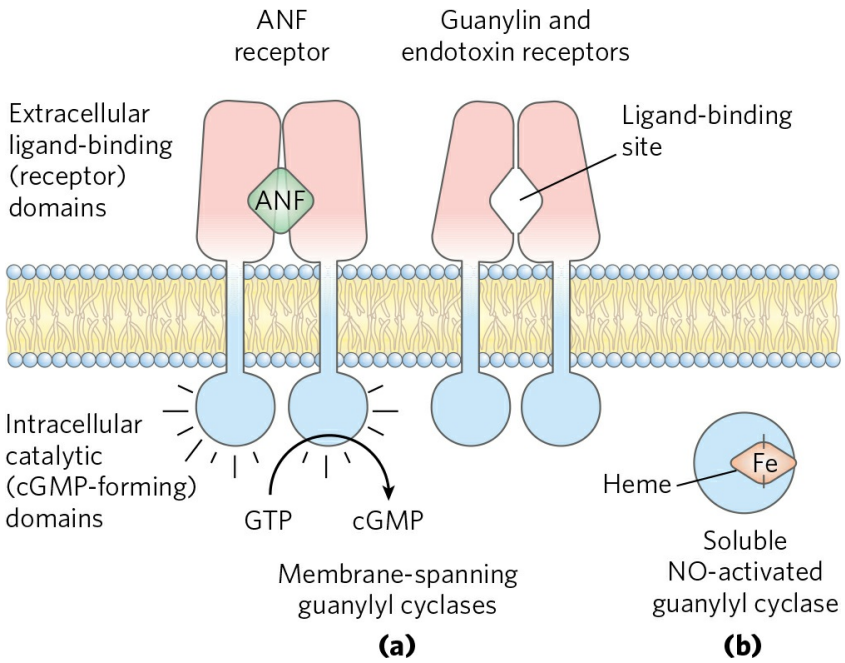


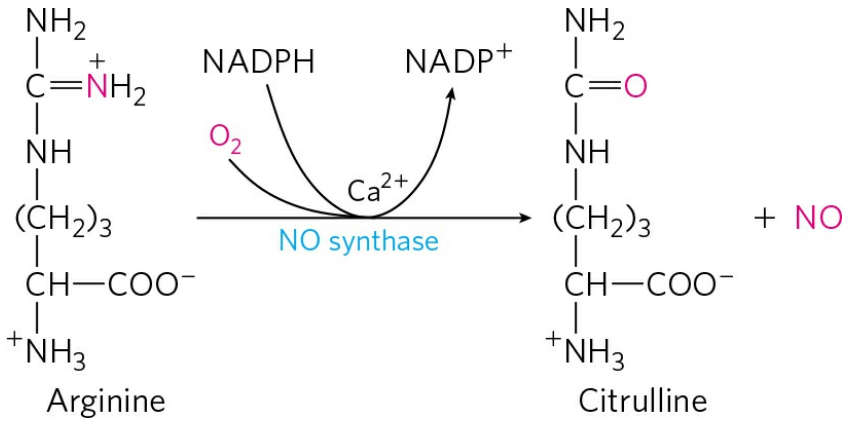
FIGURE 12-23 Two types of guanylyl cyclase that participate in signal transduction. (a) One type is a homodimer with a single membrane-spanning segment in each monomer, connecting the extracellular ligand-binding domain and the intracellular guanylyl cyclase domain. Receptors of this type are used to detect two extracellular ligands: atrial natriuretic factor (ANF; receptors in cells of the renal collecting ducts and vascular smooth muscle) and guanylin (peptide hormone produced in the intestine, with receptors in intestinal epithelial cells). The guanylin receptor is also the target of a bacterial endotoxin that triggers severe diarrhea. **(b)** The other type of guanylyl cyclase is a soluble heme-containing enzyme that is activated by intracellular nitric oxide (NO); this form is present in many tissues, including smooth muscle of the heart and blood vessels.

Cyclic GMP carries different messages in different tissues. In the kidney and intestine it triggers changes in ion transport and water retention; in cardiac muscle (a type of smooth muscle) it signals relaxation; in the brain it may be involved both in development and in adult brain function. Guanylyl cyclase in the kidney is activated by the peptide hormone **atrial natriuretic factor (ANF)**, which is released by

cells in the cardiac atrium when the heart is stretched by increased blood volume. Carried in the blood to the kidney, ANF activates guanylyl cyclase in cells of the collecting ducts (Fig. 12-23a). The resulting rise in [cGMP] triggers increased renal excretion of Na^+ and consequently of water, driven by the change in osmotic pressure. Water loss reduces the blood volume, countering the stimulus that initially led to ANF secretion. Vascular smooth muscle also has an ANF receptor–guanylyl cyclase; on binding to this receptor, ANF causes relaxation (vasodilation) of the blood vessels, which increases blood flow while decreasing blood pressure.

A similar receptor guanylyl cyclase in the plasma membrane of epithelial cells lining the intestine is activated by the peptide **guanylin** (Fig. 12-23a), which regulates Cl^- secretion in the intestine. This receptor is also the target of a heat-stable peptide endotoxin produced by *Escherichia coli* and other gram-negative bacteria. The elevation in [cGMP] caused by the endotoxin increases Cl^+ secretion and consequently decreases reabsorption of water by the intestinal epithelium, producing diarrhea.

A distinctly different type of guanylyl cyclase is a cytosolic protein with a tightly associated heme group (Fig. 12-23b), an enzyme activated by nitric oxide (NO). Nitric oxide is produced from arginine by Ca^{2+}-dependent **NO synthase**, present in many mammalian tissues, and diffuses from its cell of origin into nearby cells.

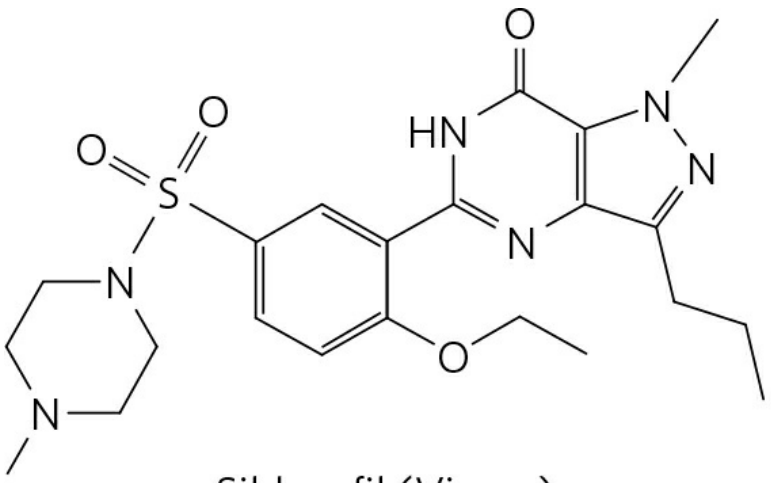


NO is sufficiently nonpolar to cross plasma membranes without a carrier. In the target cell, it binds to the heme group of guanylyl cyclase and activates cGMP production. In the heart, cGMP-dependent protein kinase reduces the forcefulness of contractions by stimulating the ion pump(s) that remove Ca^{2+} from the cytosol.

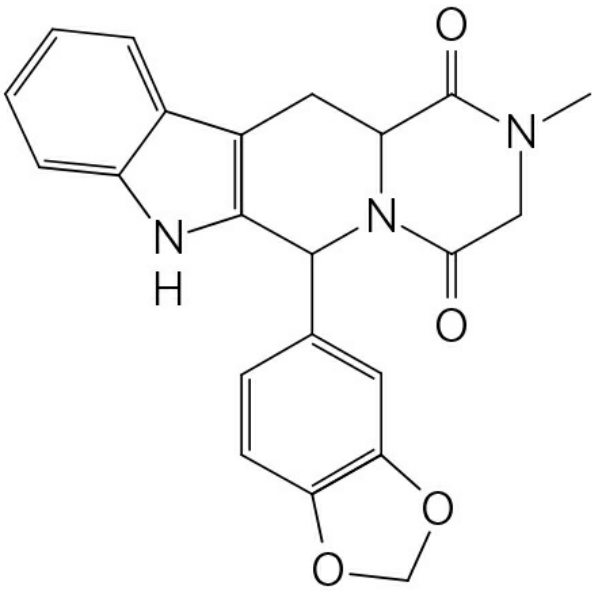
NO-induced relaxation of cardiac muscle is the same response brought about by nitroglycerin and other nitrovasodilators taken to

relieve **angina pectoris**, the pain caused by contraction of a heart deprived of O_2 because of blocked coronary arteries. Nitric oxide is unstable and its action is brief; within seconds of its formation, it undergoes oxidation to nitrite or nitrate. Nitrovasodilators produce long-lasting relaxation of cardiac muscle because they break down over several hours, yielding a steady stream of NO. The value of nitroglycerin as a treatment for angina was discovered serendipitously in factories producing nitroglycerin as an explosive in the 1860s. Workers with angina reported that their condition was much improved during the workweek but worsened on weekends. The physicians treating these workers heard this story so often that they made the connection, and a drug was born.

The effects of increased cGMP synthesis diminish after the stimulus ceases, because a specific phosphodiesterase (cGMP PDE) converts cGMP to the inactive 5′-GMP. Humans have several isoforms of cGMP PDE, with different tissue distributions. The isoform in the blood vessels of the penis is inhibited by the drugs sildenafil (Viagra) and tadalafil (Cialis), which therefore cause [cGMP] to remain elevated once raised by an appropriate stimulus, accounting for the usefulness of this drug in the treatment of erectile dysfunction.



Sildenafil (Viagra)



Tadalafil (Cialis)

Cyclic GMP has another mode of action in the vertebrate eye: it causes ion-specific channels to open in the retinal rod and cone cells, as we discussed in Section 12.3.

SUMMARY 12.5 Receptor Guanylyl Cyclases, cGMP, and Protein Kinase G

■ Several signals, including atrial natriuretic factor and guanylin, act through receptor enzymes with guanylyl cyclase activity. The cGMP so produced is a second messenger that activates cGMP-dependent protein kinase (PKG). This enzyme alters metabolism by phosphorylating specific enzyme targets.

■ Nitric oxide is a short-lived messenger that stimulates a soluble guanylyl cyclase, raising [cGMP] and stimulating PKG.

12.6 Multivalent Adaptor Proteins and Membrane Rafts

Two generalizations have emerged from studies of signaling systems such as those we have discussed so far. First, protein kinases that phosphorylate, and phosphatases that dephosphorylate, Tyr, Ser, and Thr residues are central to signaling, *directly* affecting the activities of a large number of protein substrates by phosphorylation/dephosphorylation. Second, protein-protein interactions brought about by the reversible phosphorylation of Tyr, Ser, and Thr residues in signaling proteins create *docking sites* for other proteins that bring about *indirect* effects on proteins downstream in the signaling pathway. In fact, many signaling proteins are *multivalent*: they can interact with several different proteins simultaneously to form multiprotein signaling complexes. In this section we present a few examples to illustrate the general principles of phosphorylation-dependent protein interactions in signaling pathways.

Protein Modules Bind Phosphorylated Tyr, Ser, or Thr Residues in Partner Proteins

The protein Grb2 in the insulin signaling pathway (Figs 12-19 and 12-22) binds through its SH2 domain to other proteins that have exposed Ⓟ–Tyr residues. The human genome encodes at least 87 SH2-containing proteins, many already known to participate in signaling. The Ⓟ–Tyr residue is bound in a deep pocket in an SH2 domain, with each of its phosphate oxygens participating in hydrogen bonding or electrostatic interactions; the positive charges on two Arg residues figure prominently in the binding. Subtle differences in the structure of SH2 domains account for the specificities of the interactions of SH2-containing proteins with various Ⓟ–Tyr-containing proteins. The SH2 domain typically interacts with a Ⓟ–Tyr (which is assigned the index position 0) and the next three residues toward the carboxyl terminus (designated +1, +2, +3). Some proteins with SH2 domains (Src, Fyn, Hck, Nck) favor negatively charged residues in the +1 and +2 positions; others ($PLC_\gamma 1$, SHP2) have a long hydrophobic groove that binds to aliphatic residues in positions +1 to +5. These differences define subclasses of SH2 domains that have different partner specificities.

Phosphotyrosine-binding domains, or **PTB domains**, are another binding partner for Ⓟ–Tyr proteins **(Fig. 12-24)**, but their critical

sequences and three-dimensional structure distinguish them from SH2 domains. The human genome encodes 24 proteins that contain PTB domains, including IRS-1, which we have already encountered in its role as an adaptor protein in insulin-signal transduction (Fig. 12-19). The Ⓟ–Tyr binding sites for SH2 and PTB domains on partner proteins are created by Tyr kinases and eliminated by protein tyrosine phosphatases (PTPs).

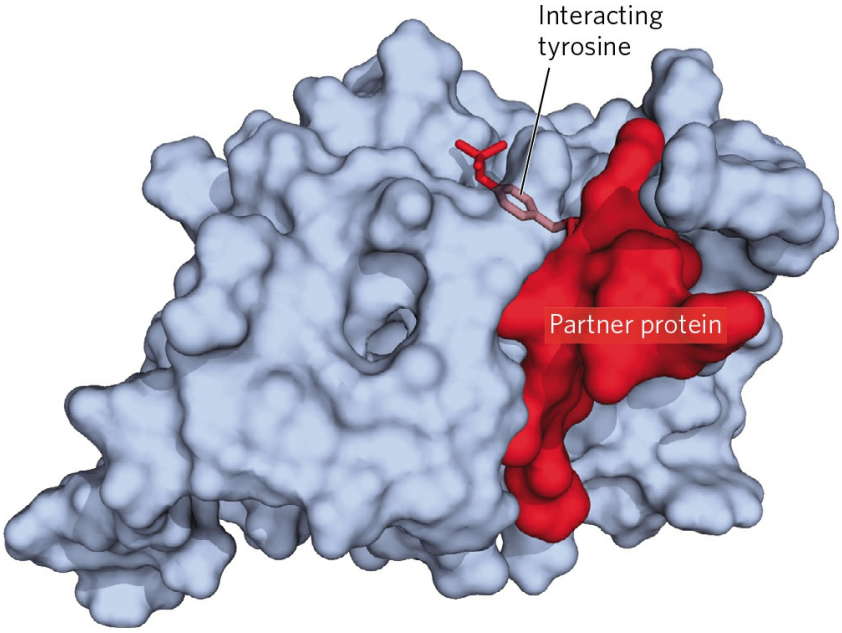


FIGURE 12-24 Interaction of a PTB domain with a Ⓟ–Tyr residue in a partner protein. The PTB domain is represented as a blue surface contour. The partner protein is held to the kinase by multiple noncovalent interactions, which confer specificity on the interaction and position the Ⓟ–Tyr residue in a binding pocket at the enzyme's active site.
[Source: PDB ID 1SHC, M. M. Zhou et al., *Nature* 378:584, 1995.]

As we have seen, other signaling protein kinases, including PKA, PKC, PKG, and members of the MAPK cascade, phosphorylate Ser or Thr residues in their target proteins, which in some cases acquire the ability to interact with partner proteins through the phosphorylated residue, triggering a downstream process. An alphabet soup of domains that bind Ⓟ–Ser or Ⓟ–Thr residues has been identified, and more are sure to be found. Each domain favors a certain sequence around the phosphorylated residue, so proteins with that domain bind to and interact with a specific subset of phosphorylated proteins.

In some cases, the region on a protein that binds Ⓟ–Tyr of a substrate protein is masked by the region's interaction with a Ⓟ–Tyr in the same protein. For example, the soluble protein Tyr kinase Src, when

phosphorylated on a critical Tyr residue, is rendered inactive; an SH2 domain needed to bind the substrate protein instead binds the internal Ⓟ–Tyr. When this Ⓟ–Tyr residue is hydrolyzed by a phosphoprotein phosphatase, the Tyr kinase activity of Src is activated **(Fig. 12-25a)**. Similarly, glycogen synthase kinase 3 (GSK3) is inactive when phosphorylated on a Ser residue in its autoinhibitory domain (Fig. 12-25b). Dephosphorylation of that domain frees the enzyme to bind (and then phosphorylate) its target proteins.

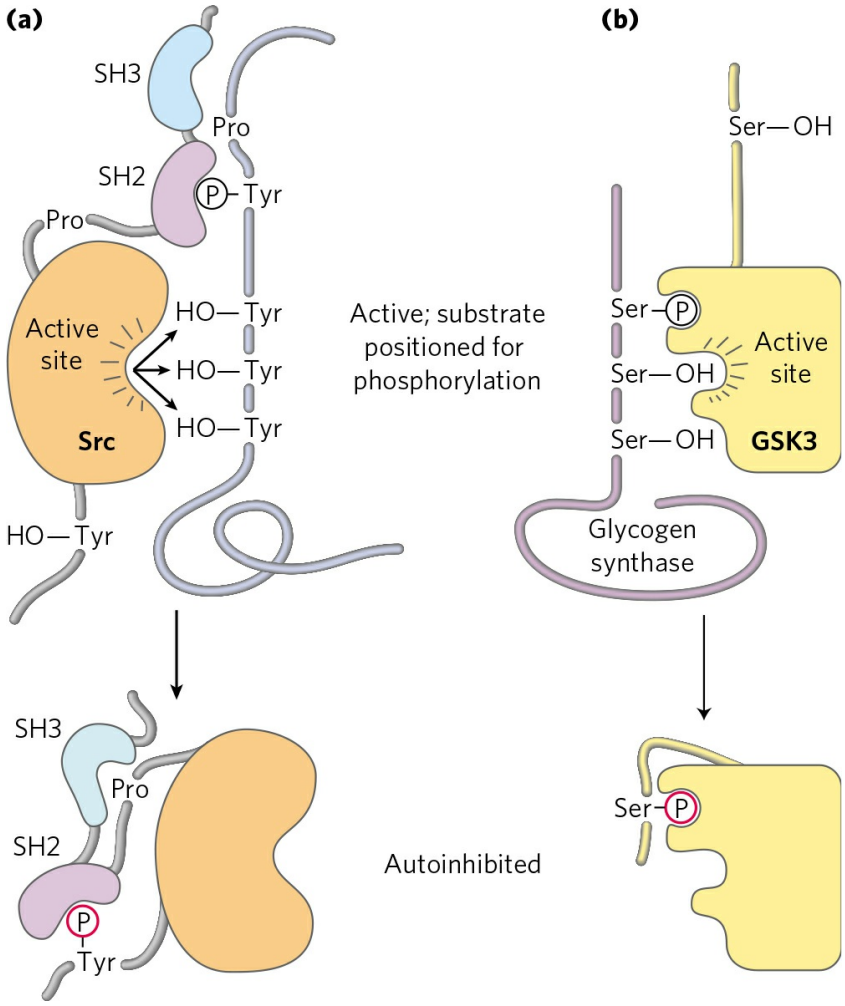


FIGURE 12-25 Mechanism of autoinhibition of Src and GSK3. (a) In the active form of the Tyr kinase Src, an SH2 domain binds a Ⓟ–Tyr in the protein substrate, and an SH3 domain binds a proline-rich region of the substrate, thus lining up the active site of the kinase with several target Tyr residues in the substrate (top). When Src is phosphorylated on a specific Tyr residue (bottom), the SH2 domain binds the internal Ⓟ–Tyr instead of the Ⓟ–Tyr of the substrate, and the SH3 domain binds an internal proline-rich region, preventing productive enzyme-substrate binding; the enzyme is thus autoinhibited. **(b)** In the active form of glycogen synthase kinase 3 (GSK3), an internal Ⓟ–Ser-

binding domain is available to bind Ⓟ–Ser in its substrate (glycogen synthase) and to position the kinase to phosphorylate neighboring Ser residues (top). Phosphorylation of an internal Ser residue allows this internal kinase segment to occupy the Ⓟ–Ser-binding site, blocking substrate binding (bottom).

In addition to the three commonly phosphorylated residues in proteins, there is a fourth phosphorylated structure that nucleates the formation of supramolecular complexes of signaling proteins: the phosphorylated head group of the membrane phosphatidylinositols. Many signaling proteins contain domains such as SH3 and PH (pleckstrin homology domain) that bind tightly to PIP_3 protruding on the cytoplasmic side of the plasma membrane. Wherever the enzyme PI3K creates this head group (as it does in response to the insulin signal), proteins that bind PIP_3 will cluster at the membrane surface.

Most of the proteins involved in signaling at the plasma membrane have one or more protein- or phospholipid-binding domains; many have three or more, and thus are multivalent in their interactions with other signaling proteins. **Figure 12-26** shows just a few of the multivalent proteins known to participate in signaling. Many of the complexes include components with membrane-binding domains. Given the location of so many signaling processes at the inner surface of the plasma membrane, the molecules that must collide to produce the signaling response are effectively confined to two-dimensional space—the membrane surface; collisions are far more likely here than in the three-dimensional space of the cytosol.

In summary, a remarkable picture of signaling pathways has emerged from studies of many signaling proteins and their multiple binding domains. An initial signal results in phosphorylation of the receptor or a target protein, triggering the assembly of large multiprotein complexes, held together on scaffolds with multivalent binding capacities. Some of these complexes contain several protein kinases that activate each other in turn, producing a cascade of phosphorylation and a great amplification of the initial signal. The interactions between cascade kinases are not left to the vagaries of random collisions in three-dimensional space. In the MAPK cascade, for example, a scaffold protein, KSR, binds all three kinases (MAPK, MAPKK, and MAPKKK), ensuring their proximity and correct orientation and even conferring allosteric properties on the interactions among the kinases, which makes their serial phosphorylation sensitive to very small stimuli **(Fig. 12-27)**.

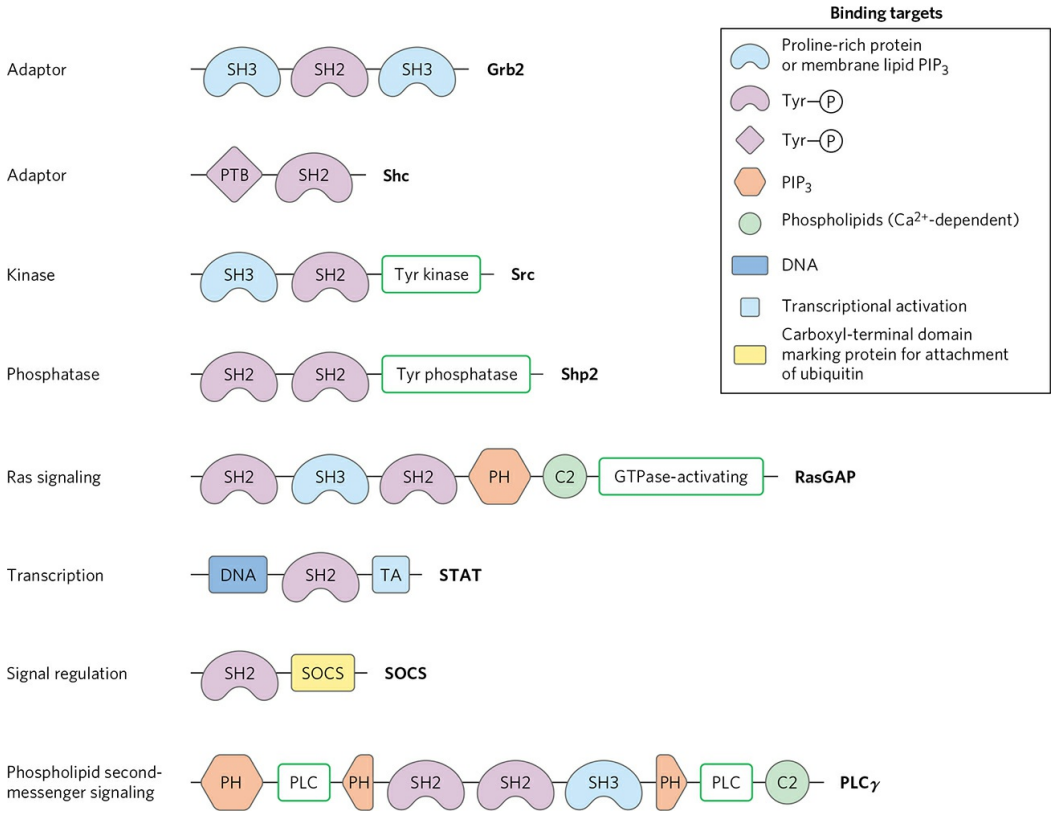


FIGURE 12-26 Some binding modules of signaling proteins. Each protein is represented by a line (with the amino terminus to the left); symbols indicate the location of conserved binding domains (with specificities as listed in the key; abbreviations are explained in the text); green boxes indicate catalytic activities. The name of each protein is given at its carboxyl-terminal end. These signaling proteins interact with phosphorylated proteins or phospholipids in many permutations and combinations to form integrated signaling complexes.
[Source: Information from T. Pawson et al., *Trends Cell Biol.* 11:504, 2001, Fig. 5.]

Phosphotyrosine phosphatases remove the phosphate from Ⓟ–Tyr residues, reversing the effect of phosphorylation. There are at least 37 genes encoding protein tyrosine phosphatases (PTPs) in the human genome. About half of these are receptorlike integral proteins with a single transmembrane domain; they are presumably controlled by extracellular factors not yet identified. Other PTPs are soluble and contain SH2 domains that determine their molecular partners and intracellular location. In addition, animal cells have protein Ⓟ–Ser and Ⓟ–Thr phosphatases such as PP1 that reverse the effects of Ser- and Thr-specific protein kinases. We can see, then, that signaling occurs in **protein circuits**, which are effectively hardwired from signal receptor to response effector and can be switched off instantly by the hydrolysis of a single upstream phosphate

ester bond. In these circuits, protein kinases are the writers, domains such as SH2 are the readers, and PTPs and other phosphatases, the erasers.

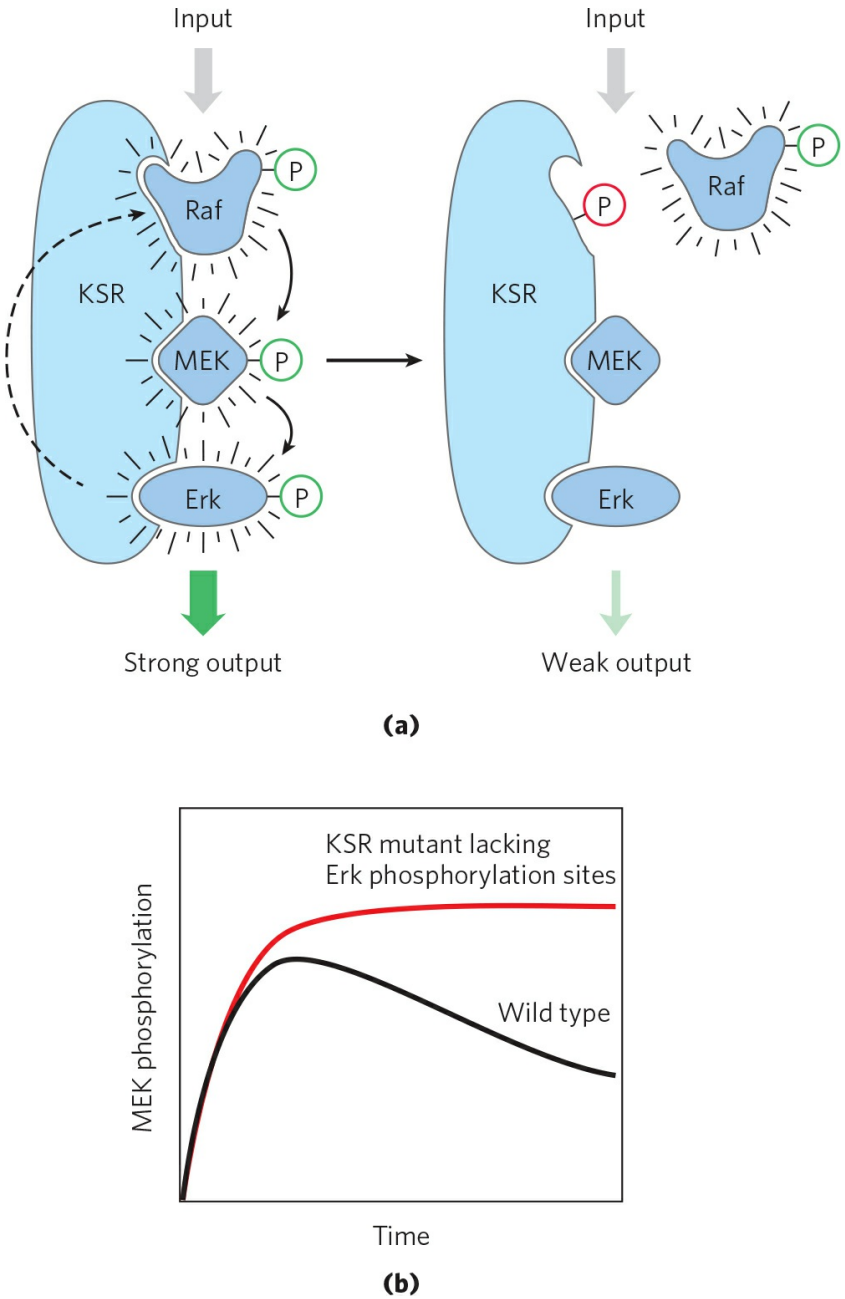


FIGURE 12-27 A scaffold protein from yeast that organizes and regulates a protein kinase cascade. (a) The scaffold protein KSR has binding sites for all three of the kinases in the Raf-MEK-Erk cascade. With the binding of all three in appropriate orientations, interactions among the proteins are rapid and efficient. When Erk has been activated (left), it phosphorylates the binding site for Raf (right), forcing a conformational change that displaces Raf and thereby prevents the phosphorylation of MEK. The result of this feedback regulation is that MEK phosphorylation is temporary. **(b)** In yeast cells with mutant KSR

lacking the phosphorylation sites (red curve), no feedback occurs, producing a different time course of signaling.
[Source: Information from M. C. Good et al., *Science* 332:680, 2011, Fig. 2E.]

The multivalency of signaling proteins allows the assembly of many different combinations of Lego-like signaling modules, each combination suited to particular signals, cell types, and metabolic circumstances, yielding diverse signaling circuits of extraordinary complexity.

Membrane Rafts and Caveolae Segregate Signaling Proteins

Membrane rafts (Chapter 11) are regions of the membrane bilayer enriched in sphingolipids, sterols, and certain proteins, including many proteins attached to the bilayer by GPI (*g*lycosylated derivatives of *p*hosphatidyl*i*nositol) anchors. The *β*-adrenergic receptor is segregated in rafts that also contain G proteins, adenylyl cyclase, PKA, and the protein phosphatase PP2, which together provide a highly integrated signaling unit. By segregating in a small region of the plasma membrane all of the elements required for responding to and ending the signal, the cell is able to produce a highly localized and brief “puff” of second messenger.

Some RTKs (EGFR and PDGFR) are also localized in rafts, and this sequestration very likely has functional significance. In isolated fibroblasts, EGFR is usually concentrated in specialized rafts called caveolae (see Fig. 11-20). When the cells are treated with EGF, the receptor leaves the raft, separating it from the other components of the EGF signaling pathway. This migration depends on the receptor’s protein kinase activity; mutant receptors lacking this activity remain in the raft during treatment with EGF. Such experiments suggest that spatial segregation of signaling proteins in rafts is yet another dimension of the already complex processes initiated by extracellular signals.

SUMMARY 12.6 Multivalent Adaptor Proteins and Membrane Rafts

■ Many signaling proteins have domains that bind phosphorylated Tyr, Ser, or Thr residues in other proteins; the binding specificity for each domain is determined by sequences that adjoin the phosphorylated residue in the substrate.

- SH2 and PTB domains bind to proteins containing Ⓟ–Tyr residues; other domains bind Ⓟ–Ser and Ⓟ–Thr residues in various contexts.
- SH3 and PH domains bind the membrane phospholipid PIP_3.
- Many signaling proteins are multivalent, with several different binding modules. By combining the substrate specificities of various protein kinases with the specificities of domains that bind phosphorylated Ser, Thr, or Tyr residues, and with phosphatases that can rapidly inactivate a signaling pathway, cells create a large number of multiprotein signaling complexes.
- Membrane rafts and caveolae sequester groups of signaling proteins in small regions of the plasma membrane, effectively raising their local concentrations and making signaling more efficient.

12.7 Gated Ion Channels

Ion Channels Underlie Electrical Signaling in Excitable Cells

Certain cells in multicellular organisms are "excitable": they can detect an external signal, convert it into an electrical signal (specifically, a change in membrane potential), and pass it on. Excitable cells play central roles in nerve conduction, muscle contraction, hormone secretion, sensory processes, and learning and memory. The excitability of sensory cells, neurons, and myocytes depends on ion channels, signal transducers that provide a regulated path for the movement of inorganic ions such as Na^+, K^+, Ca^{2+}, and Cl^- across the plasma membrane in response to various stimuli. Recall from Chapter 11 that these ion channels are "gated": they may be open or closed, depending on whether the associated receptor has been activated by the binding of its specific ligand (a neurotransmitter, for example) or by a change in the transmembrane electrical potential. The Na^+K^+ ATPase is electrogenic; it creates a charge imbalance across the plasma membrane by carrying 3 Na^+ out of the cell for every 2 K^+ carried in **(Fig. 12-28a)**. The action of the ATPase makes the inside of the cell negative relative to the outside. Inside the cell, $[K^+]$ is much higher and $[Na^+]$ is much lower than outside the cell (Fig. 12-28b). The direction of spontaneous ion flow across a polarized membrane is dictated by the electrochemical potential of that ion across the membrane, which has two components: the difference in concentration of the ion on the two sides of the membrane, and the difference in electrical potential (V_m), typically expressed in millivolts (see Eqn 11-4, p. 413). Given the ion concentration differences and a V_m of about −60 mV (inside negative), opening of a Na^+ or Ca^{2+} channel will result in a spontaneous inward flow of Na^+ or Ca^{2+} (and depolarization), whereas opening of a K^+ channel will result in a spontaneous outward flow of K^+ (and hyperpolarization) (Fig. 12-28b). In this case, K^+ moves out of the cell against the electrical gradient, because the large concentration difference exerts a stronger effect than the V_m. For Cl^-, the membrane potential predominates, so when a Cl^- channel opens, Cl^- flows outward.

The number of ions that must flow to produce a physiologically significant change in the membrane potential is negligible relative to the concentrations of Na^+, K^+, and Cl^+ in cells and extracellular fluid, so the ion fluxes that occur during signaling in excitable cells have essentially no effect on the concentrations of these ions. With Ca^{2+}, the situation is different; because the intracellular [Ca^{2+}] is generally very low (~10^{-7} M), inward flow of Ca^{2+} can significantly alter the cytosolic [Ca^{2+}], allowing it to serve as a second messenger.

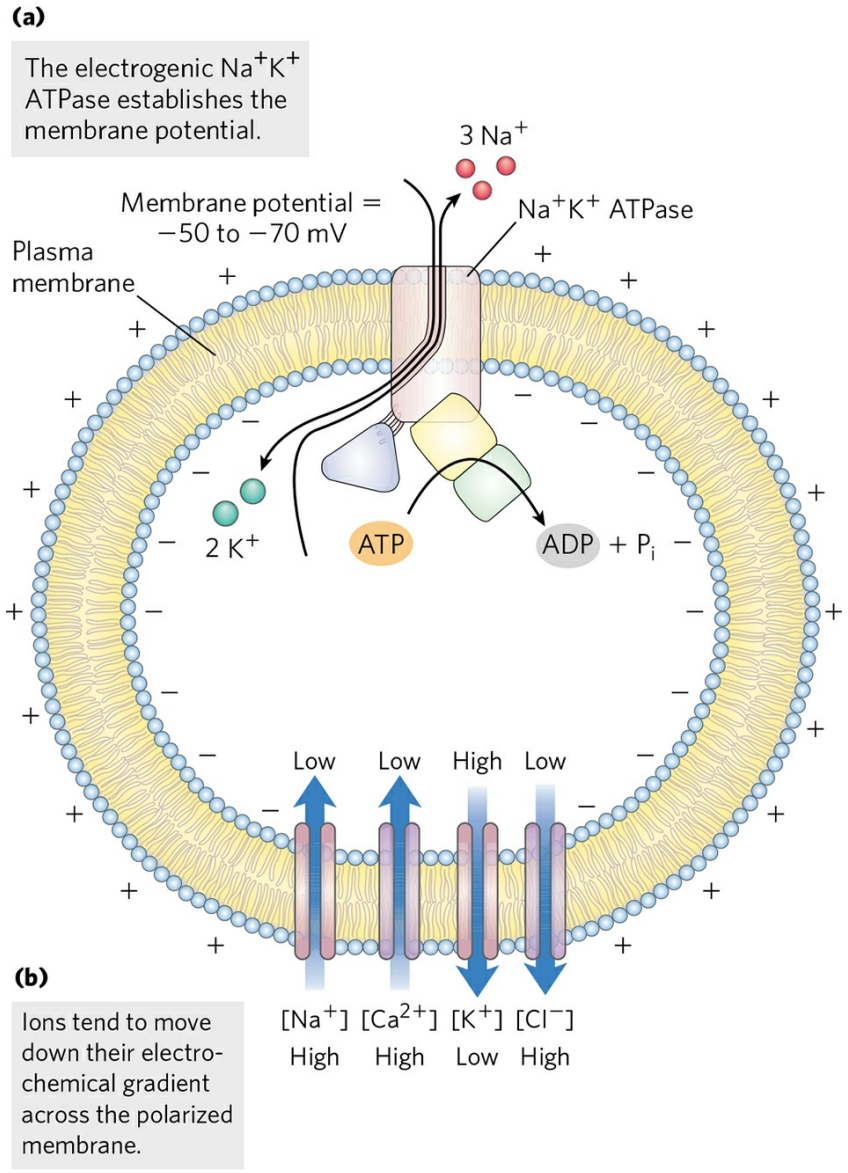


FIGURE 12-28 Transmembrane electrical potential. (a) The electrogenic Na^+K^+ ATPase produces a transmembrane electrical potential of about 60 mV (inside negative). **(b)** Blue arrows show the direction in which ions tend to move spontaneously across the plasma membrane in an animal cell, driven by the combination of chemical and

electrical gradients. The chemical gradient drives Na^+ and Ca^{2+} inward (producing depolarization) and K^+ outward, against its electrical gradient (producing hyperpolarization). The electrical gradient drives Cl^- outward, against its concentration gradient (producing depolarization).

The membrane potential of a cell at a given time is the result of the types and numbers of ion channels open at that instant. The precisely timed opening and closing of ion channels and the resulting transient changes in membrane potential underlie the electrical signaling by which the nervous system stimulates the skeletal muscles to contract, the heart to beat, or secretory cells to release their contents. Moreover, many hormones exert their effects by altering the membrane potential of their target cells. These mechanisms are not limited to animals; ion channels play important roles in the responses of bacteria, protists, and plants to environmental signals.

To illustrate the action of ion channels in cell-to-cell signaling, we describe the mechanisms by which a neuron passes a signal along its length and across a synapse to the next neuron (or to a myocyte) in a cellular circuit, using acetylcholine as the neurotransmitter.

Voltage-Gated Ion Channels Produce Neuronal Action Potentials

Signaling in the nervous system is accomplished by networks of neurons, specialized cells that carry an electrical impulse (action potential) from one end of the cell (the cell body) through an elongated cytoplasmic extension (the axon). The electrical signal triggers release of neurotransmitter molecules at the synapse, carrying the signal to the next cell in the circuit. Three types of **voltage-gated ion channels** are essential to this signaling mechanism. Along the entire length of the axon are **voltage-gated Na^+ channels** **(Fig. 12-29)**, which are closed when the membrane is at rest (V_m = −60 mV) but open briefly when the membrane is depolarized locally in response to acetylcholine (or some other neurotransmitter). Also distributed along the axon are **voltage-gated K^+ channels**, which open, a split second later, in response to the depolarization that results when nearby Na^+ channels open. The depolarizing flow of Na^+ into the axon (influx) is thus rapidly countered by a repolarizing outward flow of K^+ (efflux). At the distal end of the axon are **voltage-gated Ca_2^+ channels**, which open when the wave of depolarization (Fig. 12-29, step 1) and

repolarization (step 2) caused by the activity of Na^+ and K^+ channels arrives, triggering release of the neurotransmitter acetylcholine—which carries the signal to another neuron (fire an action potential!) or to a muscle fiber (contract!).

The voltage-gated Na^+ channels are selective for Na^+ over other cations by a factor of 100 or more. They also have a very high flux rate of $>10^7$ ions/s. A Na^+ channel that opens in response to a reduction in transmembrane electrical potential closes within milliseconds and remains unable to reopen for many milliseconds. The influx of Na^+ through the open Na^+ channels depolarizes the membrane locally, causing voltage-gated K^+ channels to open (Fig. 12-29, step 1). The resulting K^+ efflux repolarizes the membrane locally, reestablishing the inside-negative membrane potential (step 2). (We discuss the structure and mechanism of voltage-gated K^+ channels in some detail in Section 11.3; see Figs 11-45 and 11-46.) A brief pulse of depolarization thus traverses the axon as local depolarization triggers the brief opening of neighboring Na^+ channels, then K^+ channels. The short period that follows the opening of each Na^+ channel, during which it cannot open again, ensures that a unidirectional wave of depolarization—the action potential—sweeps from the nerve cell body toward the end of the axon.

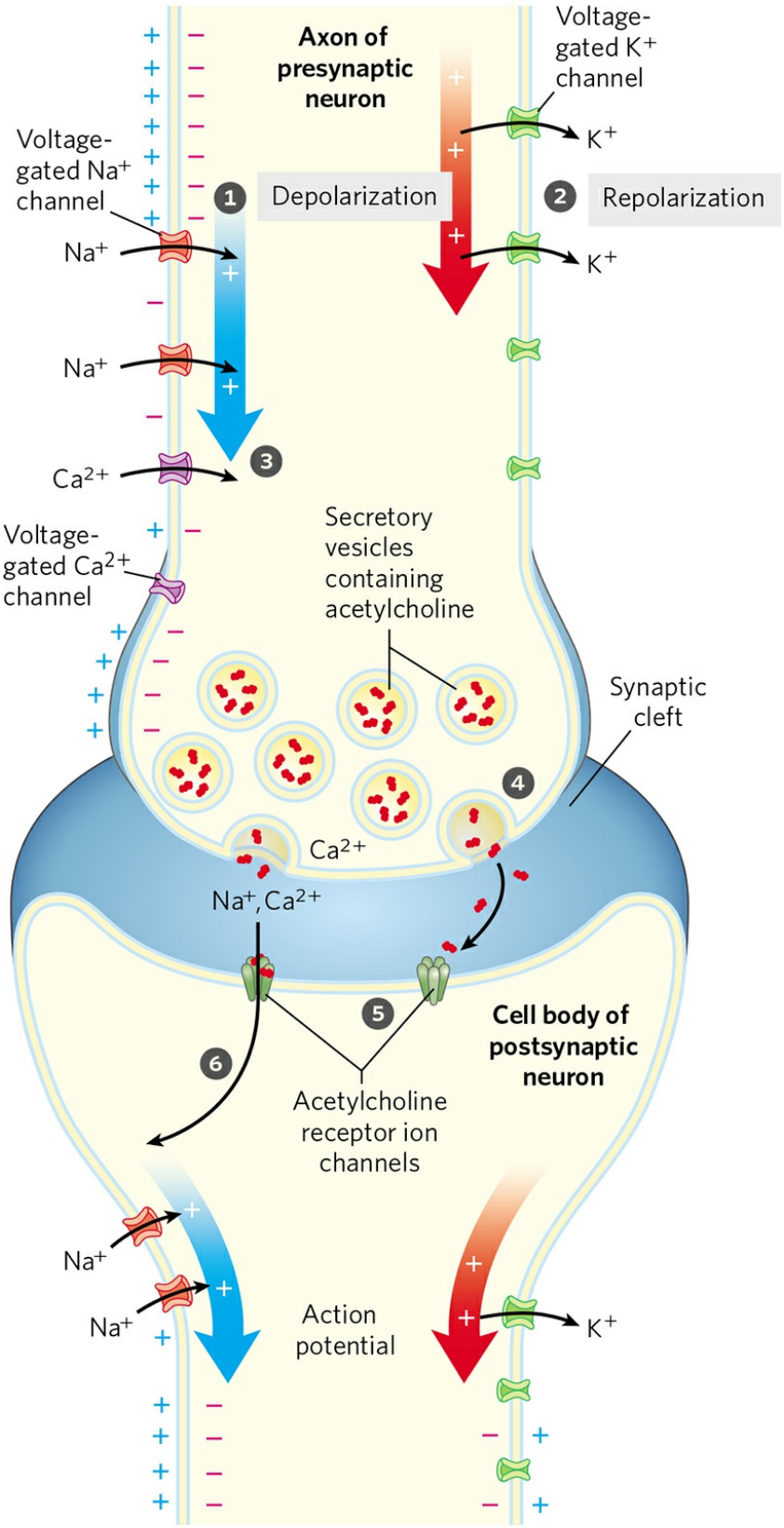


FIGURE 12-29 Role of voltage-gated and ligand-gated ion channels in neural transmission. Initially, the plasma membrane of the presynaptic neuron is polarized (inside negative) through the action of the electrogenic Na^+K^+ ATPase, which pumps out 3 Na^+ for every 2 K^+

pumped in (see Fig. 12-28). ❶ A stimulus to this neuron (not shown) causes an action potential to move along the axon (blue arrow), away from the cell body. The opening of a voltage-gated Na^+ channel allows Na^+ entry, and the resulting local depolarization causes the adjacent Na^+ channel to open, and so on. The directionality of movement of the action potential is ensured by the brief refractory period that follows the opening of each voltage-gated Na^+ channel. ❷ A split second after the action potential passes a point in the axon, voltage-gated K^+ channels open, allowing K^+ exit, which brings about repolarization of the membrane (red arrow) to make it ready for the next action potential. (Note that, for clarity, Na^+ channels and K^+ channels are drawn on opposite sides of the axon, but both types are uniformly distributed in the axonal membrane; also, positive and negative charges are shown only on the left, but as the wave of potential sweeps the axon, the membrane potential is the same at any given point along the axon.) ❸ When the wave of depolarization reaches the axon tip, voltage-gated Ca^{2+} channels open, allowing Ca^{2+} entry. ❹ The resulting increase in internal $[Ca^{2+}]$ triggers exocytotic release of the neurotransmitter acetylcholine into the synaptic cleft. ❺ Acetylcholine binds to a receptor on the postsynaptic neuron (or myocyte), causing its ligand-gated ion channel to open. ❻ Extracellular Na^+ and Ca^{2+} enter through this channel, depolarizing the postsynaptic cell. The electrical signal has thus passed to the cell body of the postsynaptic neuron and will move along its axon to a third neuron (or a myocyte) by this same sequence of events.

When the wave of depolarization reaches the voltage-gated Ca^{2+} channels, they open (step ❸), and Ca^{2+} enters from the extracellular space. The rise in cytoplasmic $[Ca^{2+}]$ then triggers release of acetylcholine by exocytosis into the synaptic cleft (step ❹). Acetylcholine diffuses to the postsynaptic cell (another neuron or a myocyte), where it binds to acetylcholine receptors and triggers depolarization (described below). Thus the message is passed to the next cell in the circuit. We see, then, that gated ion channels convey signals in either of two ways: by changing the cytoplasmic concentration of an ion (such as Ca^{2+}), which then serves as an intracellular second messenger, or by changing V_m and affecting other membrane proteins that are sensitive to V_m. The passage of an electrical signal through one neuron and on to the next illustrates both types of mechanism.

Neurons Have Receptor Channels That Respond to Different Neurotransmitters

Animal cells, especially those of the nervous system, contain a variety of ion channels gated by ligands, voltage, or both. Receptors that are themselves ion channels are classified as **ionotropic**, to distinguish them from receptors that generate a second messenger (**metabotropic** receptors). Acetylcholine acts on an ionotropic receptor in the postsynaptic cell. The acetylcholine receptor is a cation channel. When occupied by acetylcholine, the receptor opens to the passage of cations (Na^+, K^+, and Ca^{2+}), triggering depolarization of the cell. The neurotransmitters serotonin, glutamate, and glycine all can act through ionotropic receptors that are structurally related to the acetylcholine receptor. Serotonin and glutamate trigger the opening of cation (Na^+, K^+, Ca^{2+}) channels, whereas glycine opens Cl^--specific channels.

Depending on which ion passes through a channel, binding of the ligand (neurotransmitter) for that channel results in either depolarization or hyperpolarization of the target cell. A single neuron normally receives input from many other neurons, each releasing its own characteristic neurotransmitter with its characteristic depolarizing or hyperpolarizing effect. The target cell's V_m therefore reflects the *integrated* input (Fig. 12-1e) from multiple neurons. The cell responds with an action potential only if the integrated input adds up to a net depolarization of sufficient size.

The receptor channels for acetylcholine, glycine, glutamate, and γ-aminobutyric acid (GABA) are gated by *extracellular* ligands. *Intracellular* second messengers—such as cAMP, cGMP, IP_3, Ca^{2+}, and ATP—regulate ion channels of the type we saw in the sensory transductions of vision, olfaction, and gustation.

Toxins Target Ion Channels

Many of the most potent toxins found in nature act on ion channels. For example, dendrotoxin (from the black mamba snake) blocks the action of voltage-gated K^+ channels, tetrodotoxin (produced by puffer fish) acts on voltage-gated Na^+ channels, and cobrotoxin disables acetylcholine receptor ion channels. Why, in the course of evolution, have ion channels become the preferred target of toxins, rather than some critical metabolic target such as an enzyme essential in energy metabolism?

Ion channels are extraordinary amplifiers; opening of a single channel can allow the flow of 10 million ions per second. Consequently, relatively few molecules of an ion channel protein are needed per neuron for signaling functions. This means that a relatively small number of toxin molecules with high affinity for ion channels, acting from outside the cell, can have a pronounced effect on neurosignaling throughout the body. A comparable effect by way of a metabolic enzyme, typically present in cells at much higher concentrations than ion channels, would require far greater numbers of the toxin molecule.

SUMMARY 12.7 Gated Ion Channels

- Ion channels gated by membrane potential or ligands are central to signaling in neurons and other cells.
- The voltage-gated Na^+ and K^+ channels of neuronal membranes carry the action potential along the axon as a wave of depolarization (Na^+ influx) followed by repolarization (K^+ efflux).
- Arrival of an action potential at the distal end of a presynaptic neuron triggers neurotransmitter release. The neurotransmitter (acetylcholine, for example) diffuses to the postsynaptic neuron (or the myocyte, at a neuromuscular junction), binds to specific receptors in the plasma membrane, and triggers a change in V_m.
- Neurotoxins, produced by many organisms, attack neuronal ion channels and are therefore fast-acting and deadly.

12.8 Regulation of Transcription by Nuclear Hormone Receptors

The steroid, retinoic acid (retinoid), and thyroid hormones form a large group of receptor ligands that exert at least part of their effects by a mechanism fundamentally different from that of other hormones: they act directly in the nucleus to alter gene expression. We discuss their mode of action in detail in Chapter 28, along with other mechanisms for regulating gene expression. Here we give a brief overview.

Steroid hormones (estrogen, progesterone, and cortisol, for example), too hydrophobic to dissolve readily in the blood, are transported on specific carrier proteins from their point of release to their target tissues. In target cells, these hormones pass through the plasma membrane and nuclear membrane by simple diffusion and bind to specific receptor proteins in the nucleus **(Fig. 12-30)**. Hormone binding triggers changes in the conformation of a receptor protein so that it becomes capable of interacting with specific regulatory sequences in DNA called **hormone response elements (HREs)**, thus altering gene expression (see Fig. 28-33). The bound receptor-hormone complex enhances the expression of specific genes adjacent to HREs, with the help of several other proteins essential for transcription. Hours or days are required for these regulators to have their full effect—the time required for the changes in RNA synthesis and subsequent protein synthesis to become evident in altered metabolism.

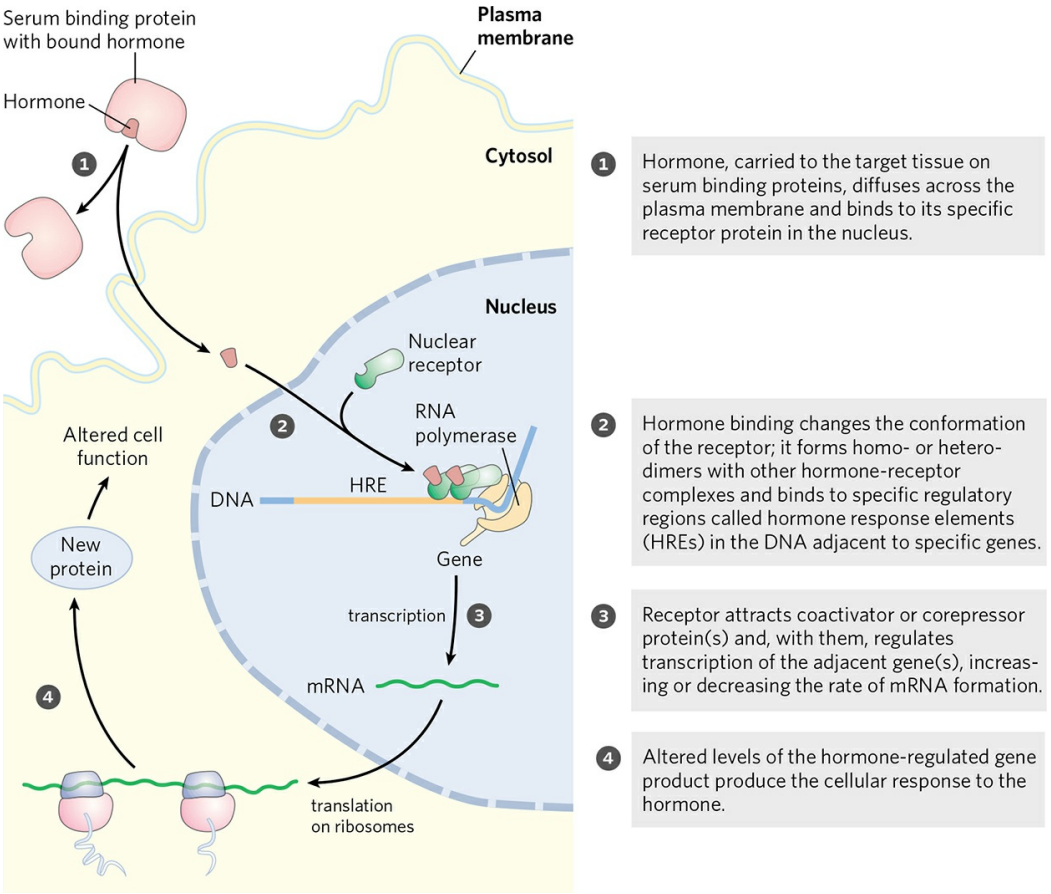
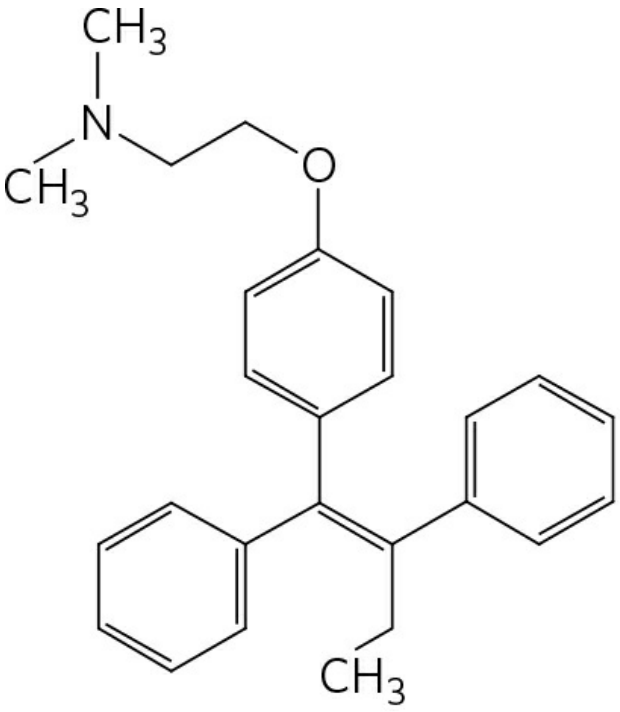
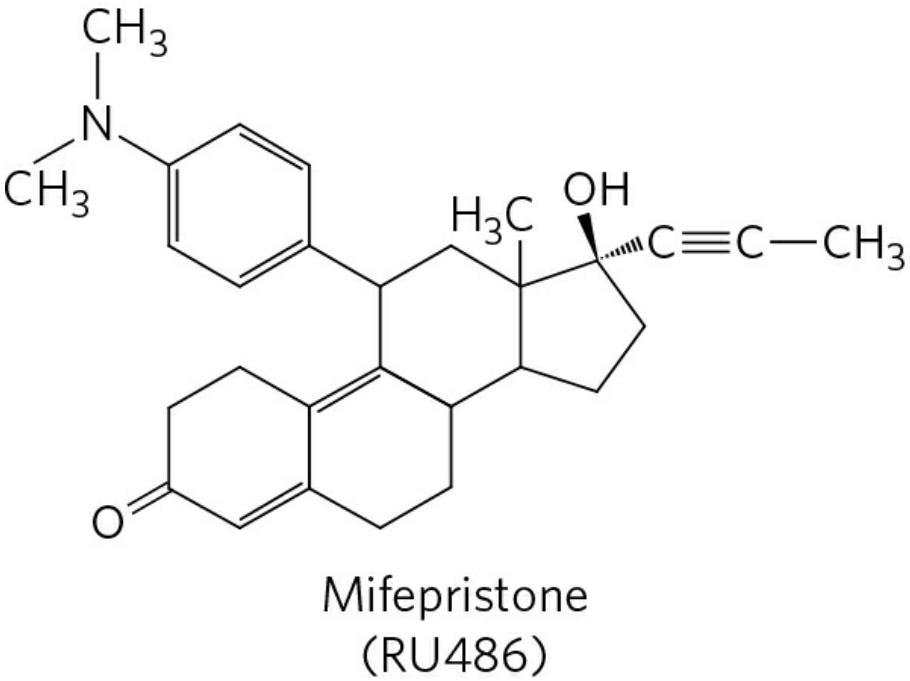


FIGURE 12-30 General mechanism by which steroid and thyroid hormones, retinoids, and vitamin D regulate gene expression. The details of transcription and protein synthesis are discussed in Chapters 26 and 27. Some steroids also act through plasma membrane receptors by a completely different mechanism.

The specificity of the steroid-receptor interaction is exploited in the use of the drug **tamoxifen** to treat breast cancer. In some types of breast cancer, division of the cancerous cells depends on the continued presence of estrogen. Tamoxifen is an estrogen antagonist; it competes with estrogen for binding to the estrogen receptor, but the tamoxifen-receptor complex has little or no effect on gene expression. Consequently, tamoxifen administered after surgery or during chemotherapy for hormone-dependent breast cancer slows or stops the growth of remaining cancerous cells. Another steroid analog, the drug **mifepristone (RU486)**, binds to the progesterone receptor and blocks hormone actions essential to implantation of the fertilized ovum in the uterus, and thus functions as a contraceptive.



Tamoxifen



Mifepristone
(RU486)

SUMMARY 12.8 Regulation of Transcription by Nuclear Hormone Receptors

■ Steroid hormones enter cells by simple diffusion and bind to specific receptor proteins.

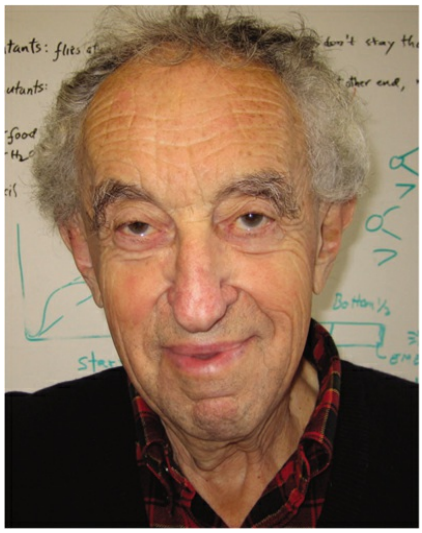
- The hormone-receptor complex binds specific regions of DNA, the hormone response elements, and interacts with other proteins to regulate the expression of nearby genes.

12.9 Signaling in Microorganisms and Plants

Much of what we have said about signaling relates to mammalian tissues or cultured cells from such tissues. Bacteria, archaea, eukaryotic microorganisms, and vascular plants must also respond to a variety of external signals—O_2, nutrients, light, noxious chemicals, and so on. We turn here to a brief consideration of the kinds of signaling machinery used by microorganisms and plants.

Bacterial Signaling Entails Phosphorylation in a Two-Component System

In pioneering studies of chemotaxis in bacteria, Julius Adler showed that *E. coli* responds to nutrients in its environment, including sugars and amino acids, by swimming toward them, propelled by surface flagella. A family of membrane proteins have binding domains on the outside of the plasma membrane to which specific **attractants** (sugars or amino acids) bind **(Fig. 12-31)**. The signal is transmitted by the so-called **two-component system.** The first component is a **receptor histidine kinase** that, in response to ligand binding, phosphorylates a His residue in its cytoplasmic domain, then catalyzes transfer of the phosphoryl group from the His residue to an Asp residue on the second component, a soluble protein called the **response regulator**. This phosphoprotein moves to the base of a flagellum, carrying the signal from the membrane receptor. Each flagellum is driven by a rotary motor that can propel the cell through its medium or cause it to stall, depending on the direction of motor rotation. The change in attractant concentration over time, signaled through the receptor, allows the cell to determine whether it is moving toward or away from the attractant. If its motion is toward the attractant, the response regulator signals the cell to continue in a straight line (a run); if away from it, the cell tumbles momentarily, acquiring a new direction. Repetition of this behavior results in a random path, biased toward movement in the direction of increasing attractant concentration.



Julius Adler
[Source: Courtesy Hildegard Wohl Adler.]

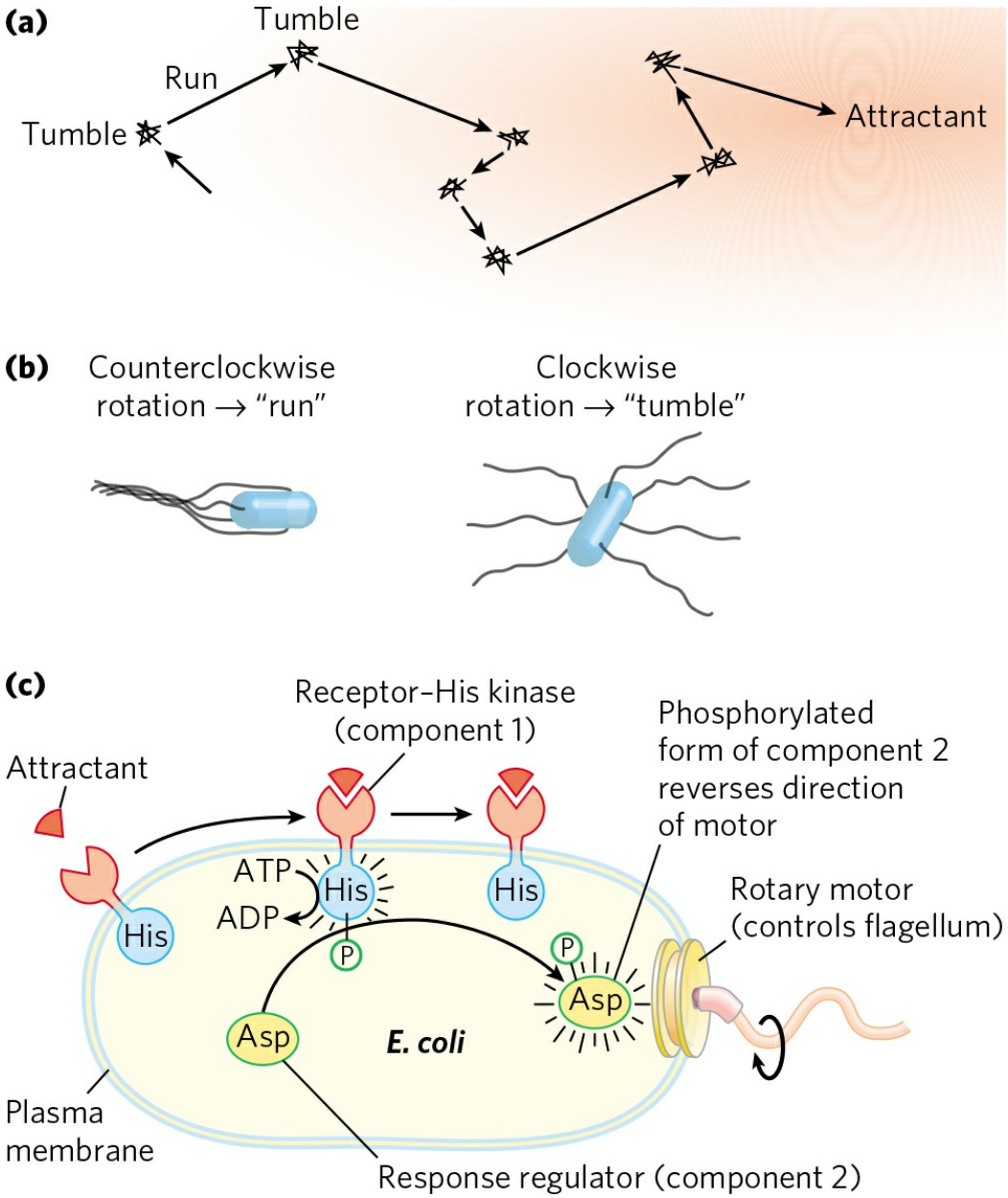


FIGURE 12-31 The two-component signaling mechanism in bacterial chemotaxis. (a) When placed near a source of an attractant solute, *E. coli* performs a random walk, biased toward the attractant. **(b)** Flagella have intrinsic helical structure, and when all flagella rotate counterclockwise, the flagellar helices twist together and move in concert to propel the cell forward in a "run." When the flagella rotate clockwise, the flagellar bundles fly apart, and the cell tumbles briefly until counterclockwise rotation resumes and the cell begins to swim forward again in a new, random direction. When moving toward the attractant, the cell has fewer tumbles and therefore longer runs; when moving away, the frequent tumbles eventually result in movement toward the attractant. **(c)** Flagellar rotation is controlled by a two-component system consisting of a receptor-histidine kinase and an effector protein. When an attractant ligand binds to the receptor domain

of the membrane-bound receptor, a protein kinase in the cytosolic domain (component 1) is activated and autophosphorylates a His residue. This phosphoryl group is then transferred to an Asp residue on a response regulator (component 2). After phosphorylation, the response regulator moves to the base of the flagellum, where it causes counterclockwise rotation of the flagella, producing a run.

E. coli detects not only sugars and amino acids but also O_2, extremes of temperature, and other environmental factors, using this basic two-component system. Two-component systems have been detected in many other bacteria, both gram-positive and gram-negative, and in archaea, as well as in protists and fungi. Clearly, this signaling mechanism developed early in the course of cellular evolution and has been conserved.

Various signaling systems used by animal cells also have analogs in bacteria. As the full genomic sequences of more, and increasingly diverse, bacteria become known, researchers have discovered genes that encode proteins similar to protein Ser or Thr kinases, Ras-like proteins regulated by GTP binding, and proteins with SH3 domains. Receptor Tyr kinases have not been detected in bacteria, but Ⓟ–Tyr residues do occur in some bacteria.

Signaling Systems of Plants Have Some of the Same Components Used by Microbes and Mammals

Like animals, vascular plants must have a means of communication between tissues to coordinate and direct growth and development, to adapt to conditions of O_2, nutrients, light, temperature, and water availability, and to warn of the presence of noxious chemicals and damaging pathogens. At least a billion years of evolution have passed since the plant and animal branches of the eukaryotes diverged, which is reflected in the differences in signaling mechanisms: some plant mechanisms are conserved—that is, are similar to those in animals (protein kinases, adaptor proteins, cyclic nucleotides, electrogenic ion pumps, and gated ion channels); some are similar to bacterial two-component systems; and some are unique to plants (such as light-sensing mechanisms that reflect seasonal changes in the angle, and hence color, of sunlight). The genome of the plant *Arabidopsis thaliana* encodes about 1,000 protein Ser/Thr kinases, including about 60 MAPKs and nearly 400 membrane-associated receptor kinases that phosphorylate Ser or Thr residues; a variety of protein phosphatases; enzymes for the synthesis and degradation of cyclic nucleotides; and 100 or more ion channels, including about 20 gated by

cyclic nucleotides. Inositol phospholipids are present, as are kinases that interconvert them by phosphorylation of inositol head groups. Even given that *Arabidopsis* has multiple copies of many genes, the presence of this many signaling-related genes certainly reflects a wide array of signaling potential.

Some types of signaling proteins common in animal tissues are not present in plants, or are represented by only a few genes. Protein kinases that are activated by cyclic nucleotides (PKA and PKG) seem to be absent, for example. Heterotrimeric G protein and protein Tyr kinase genes are much less prominent in the plant genome, and the mode of action of these proteins is different from that in animal cells. GPCRs, the largest family of signaling proteins in the human genome, are absent from the plant genome. DNA-binding nuclear steroid receptors are certainly not prominent, and may be absent from plants. Although vascular plants lack the most widely conserved light-sensing mechanism present in animals (rhodopsin, with retinal as pigment), they have a rich collection of other light-detecting mechanisms not found in animal tissues—phytochromes and cryptochromes, for example (Chapter 20).

SUMMARY 12.9 Signaling in Microorganisms and Plants

- Bacteria and eukaryotic microorganisms have a variety of sensory systems that allow them to sample and respond to their environment. In the two-component system, a receptor His kinase senses the signal and autophosphorylates a His residue, then phosphorylates an Asp residue of the response regulator.
- Plants respond to many environmental stimuli and employ hormones and growth factors to coordinate the development and metabolic activities of their tissues. Plant genomes encode hundreds of signaling proteins, including some very similar to those of mammals.
- Plants do not have GPCRs or protein kinases activated by cAMP or cGMP.

12.10 Regulation of the Cell Cycle by Protein Kinases

One of the most dramatic manifestations of signaling pathways is the regulation of the eukaryotic cell cycle. During embryonic growth and later development, cell division occurs in virtually every tissue. In the adult organism, most tissues become quiescent. A cell's "decision" to divide or not is of crucial importance to the organism. When the regulatory mechanisms that limit cell division are defective and cells undergo unregulated division, the result is catastrophic—cancer. Proper cell division requires a precisely ordered sequence of biochemical events that assures every daughter cell a full complement of the molecules required for life. Investigations into the control of cell division in diverse eukaryotic cells have revealed universal regulatory mechanisms. Signaling mechanisms much like those discussed above are central in determining whether and when a cell undergoes cell division, and they also ensure orderly passage through the stages of the cell cycle.

The Cell Cycle Has Four Stages

Cell division accompanying mitosis in eukaryotes occurs in four well-defined stages **(Fig. 12-32)**. In the S (synthesis) phase, the DNA is replicated to produce copies for both daughter cells. In the G2 phase (*G* indicates the gap between divisions), new proteins are synthesized and the cell approximately doubles in size. In the M phase (mitosis), the maternal nuclear envelope breaks down, paired chromosomes are pulled to opposite poles of the cell, each set of daughter chromosomes is surrounded by a newly formed nuclear envelope, and cytokinesis pinches the cell in half, producing two daughter cells (see Fig. 24-23). In embryonic or rapidly proliferating tissue, each daughter cell divides again, but only after a waiting period (G1). In cultured animal cells the entire process takes about 24 hours.

After passing through mitosis and into G1, a cell either continues through another division or ceases to divide, entering a quiescent phase (G0) that may last hours, days, or the lifetime of the cell. When a cell in G0 begins to divide again, it reenters the division cycle through the G1 phase. Differentiated cells such as hepatocytes or adipocytes have acquired their specialized function and form; they remain in the G0 phase. Stem

cells retain their potential to divide and to differentiate into any of a number of cell types.

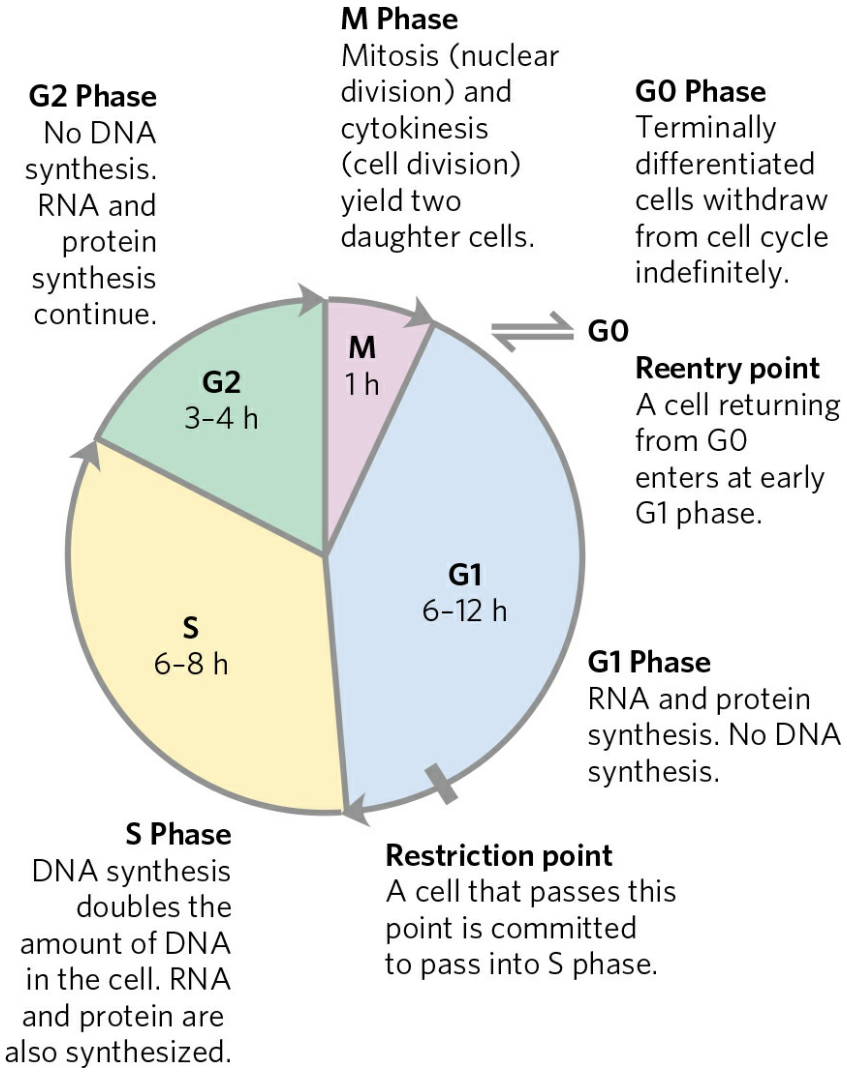


FIGURE 12-32 The eukaryotic cell cycle. The durations (in hours) of the four stages vary, but those shown are typical.

Levels of Cyclin-Dependent Protein Kinases Oscillate

The timing of the cell cycle is controlled by a family of protein kinases with activities that change in response to cellular signals. By phosphorylating specific proteins at precisely timed intervals, these protein kinases orchestrate the metabolic activities of the cell to produce orderly cell division. The kinases are heterodimers with a regulatory subunit, a **cyclin**, and a catalytic subunit, a **cyclin-dependent protein kinase (CDK)**. In the absence of the cyclin, the catalytic subunit is virtually inactive. When the cyclin binds, the catalytic site opens up, a residue essential to catalysis becomes accessible, and the protein kinase activity of

the catalytic subunit increases 10,000-fold. Animal cells have at least 10 different cyclins (designated A, B, and so forth) and at least 8 CDKs (CDK1 through CDK8), which act in various combinations at specific points in the cell cycle. Plants also use a family of CDKs to regulate their cell division in root and shoot meristems, the principal tissues in which division occurs.

In a population of animal cells undergoing synchronous division, some CDK activities show striking oscillations **(Fig. 12-33)**. These oscillations are the result of four mechanisms for regulating CDK activity: phosphorylation or dephosphorylation of the CDK, controlled degradation of the cyclin subunit, periodic synthesis of CDKs and cyclins, and the action of specific CDK-inhibiting proteins. The precisely timed activation and inactivation of a series of CDKs produces signals serving as a master clock that orchestrates the events in normal cell division and ensures that one stage is completed before the next begins.

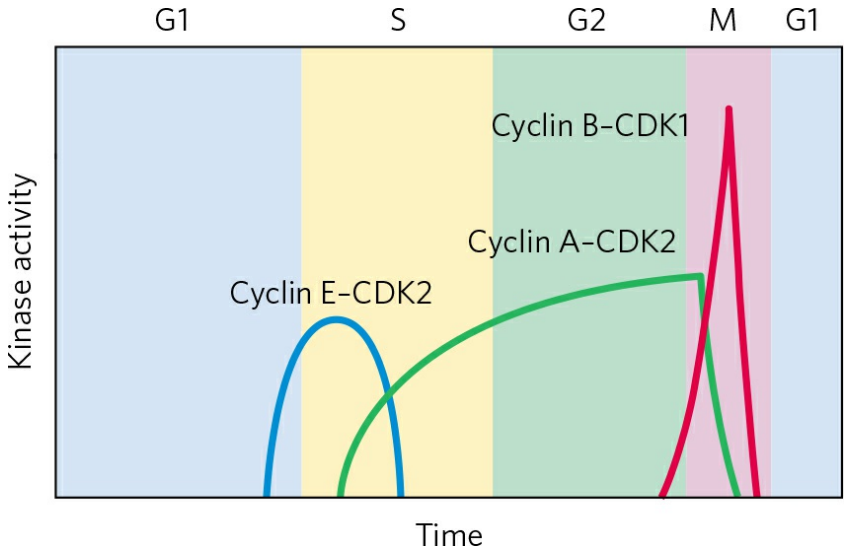


FIGURE 12-33 Variations in the activities of specific CDKs during the cell cycle in animals. Cyclin E–CDK2 activity peaks near the G1 phase–S phase boundary, when the active enzyme triggers synthesis of enzymes required for DNA synthesis (see Fig. 12-37). Cyclin A–CDK2 activity rises during the S and G2 phases, then drops sharply in the M phase, as cyclin B–CDK1 peaks. Cyclin D is active as long as a growth factor is present (not shown).
[Source: Data from J. Pines, *Nature Cell Biol.* 1:E73, 1999.]

Regulation of CDKs by Phosphorylation The activity of a CDK is strikingly affected by phosphorylation and dephosphorylation of two critical residues in the protein **(Fig. 12-34)**. Phosphorylation of Thr^{160} of CDK2 stabilizes a conformation in which an autoinhibitory "T loop" is

moved away from the substrate-binding cleft in the kinase, opening it to bind protein substrates. Dephosphorylation of Ⓟ-Tyr^{15} of CDK2 removes a negative charge that blocks ATP from approaching its binding site. This mechanism for activating a CDK is self-reinforcing; the enzyme (PTP) that dephosphorylates Ⓟ-Tyr^{15} is itself a substrate for the CDK and is activated by phosphorylation. The combination of these factors activates the CDK manyfold, allowing it to phosphorylate downstream protein targets critical to progression of the cell cycle **(Fig. 12-35a)**.

The presence of a single-strand break in DNA signals arrest of the cell cycle in G2 by activating two proteins (ATM and ATR; see Fig. 12-37). These proteins trigger a cascade of responses that include inactivation of the PTP that dephosphorylates Tyr^{15} of the CDK. With the CDK inactivated, the cell is arrested in G2, unable to divide until the DNA is repaired and the effects of the cascade are reversed.

Controlled Degradation of Cyclin Highly specific and precisely timed proteolytic breakdown of mitotic cyclins regulates CDK activity throughout the cell cycle (Fig. 12-35b). Progress through mitosis requires first the activation then the destruction of cyclins A and B, which activate the catalytic subunit of the M-phase CDK. These cyclins contain near their amino terminus the sequence –Arg–Thr–Ala–Leu–Gly–Asp–Ile–Gly–Asn–, the "destruction box," which targets the proteins for degradation. (This usage of "box" derives from the common practice, in diagramming the sequence of a nucleic acid or protein, of enclosing within a box a short sequence of nucleotide or amino acid residues with some specific function. It does not imply any three-dimensional structure.) The protein DBRP (destruction box recognizing protein) recognizes this sequence and initiates the process of cyclin degradation by bringing together the cyclin and another protein, **ubiquitin**. The cyclin and activated ubiquitin are covalently joined by the enzyme ubiquitin ligase. Several more ubiquitin molecules are then appended, providing the signal for a proteolytic enzyme complex, or **proteasome**, to degrade cyclin.

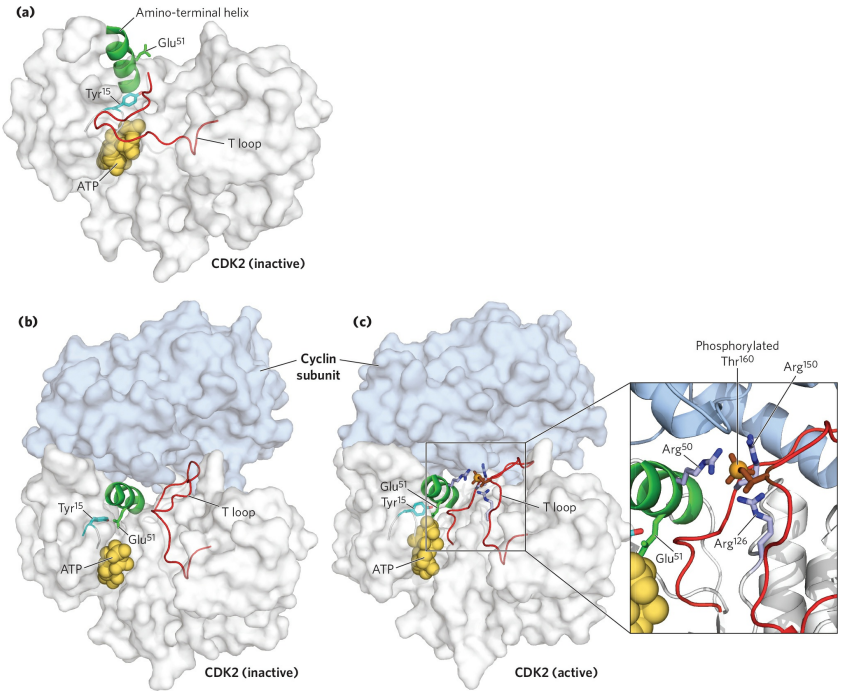


FIGURE 12-34 Activation of cyclin-dependent protein kinases (CDKs) by cyclin and phosphorylation. CDKs are active only when associated with a cyclin. The crystal structure of CDK2 with and without a cyclin reveals the basis for this activation. **(a)** Without the cyclin, CDK2 folds so that one segment, the T loop, obstructs the binding site for protein substrates. The binding site for ATP is also near the T loop and is blocked when Tyr^{15} is phosphorylated (not shown). **(b)** When the cyclin binds, it forces conformational changes that move the T loop away from the active site and reorient an amino-terminal helix, bringing a residue critical to catalysis (Glu^{51}) into the active site. **(c)** When a Thr residue in the T loop is phosphorylated, its negative charges are stabilized by interaction with three Arg residues, holding the T loop away from the substrate-binding site. Removal of the phosphoryl group on Tyr^{15} gives ATP access to its binding site, fully activating CDK2 (see Fig. 12-35). [Sources: (a) PDB ID 1HCK, U. Schulze-Gahmen et al., *J. Med. Chem.* 39:4540, 1996. (b) PDB ID 1FIN, P. D. Jeffrey et al., *Nature* 376:313, 1995. (c) PDB ID 1JST, A. A. Russo et al., *Nature Struct. Biol.* 3:696, 1996.]

How is the timing of cyclin breakdown controlled? A feedback loop occurs in the overall process shown in Figure 12-35. Increased CDK activity (step 4) leads, eventually, to cyclin proteolysis (step 8). Newly synthesized cyclin associates with and activates the CDK, which phosphorylates and activates DBRP. Active DBRP then causes proteolysis of the cyclin. The lowered cyclin level causes a decline in CDK activity, and the activity of DBRP also drops through slow, constant

dephosphorylation and inactivation by a DBRP phosphatase. The cyclin level is ultimately restored by synthesis of new cyclin molecules.

The role of ubiquitin and proteasomes is not limited to the regulation of cyclins; as we shall see in Chapter 27, both also take part in the turnover of cellular proteins, a process fundamental to cellular housekeeping.

Growth Factors Stimulate CDK and Cyclin Synthesis The third mechanism for changing CDK activity is regulation of the rate of synthesis of the cyclin or CDK or both. Extracellular signals such as **growth factors** and cytokines (developmental signals that trigger cell division) activate, by phosphorylation, the nuclear transcription factors Jun and Fos, which promote the synthesis of many gene products, including cyclins, CDKs, and the transcription factor E2F. In turn, E2F stimulates production of several enzymes essential for the synthesis of deoxynucleotides and DNA, and the CDK and cyclin allow the cell to enter the S phase **(Fig. 12-36)**.

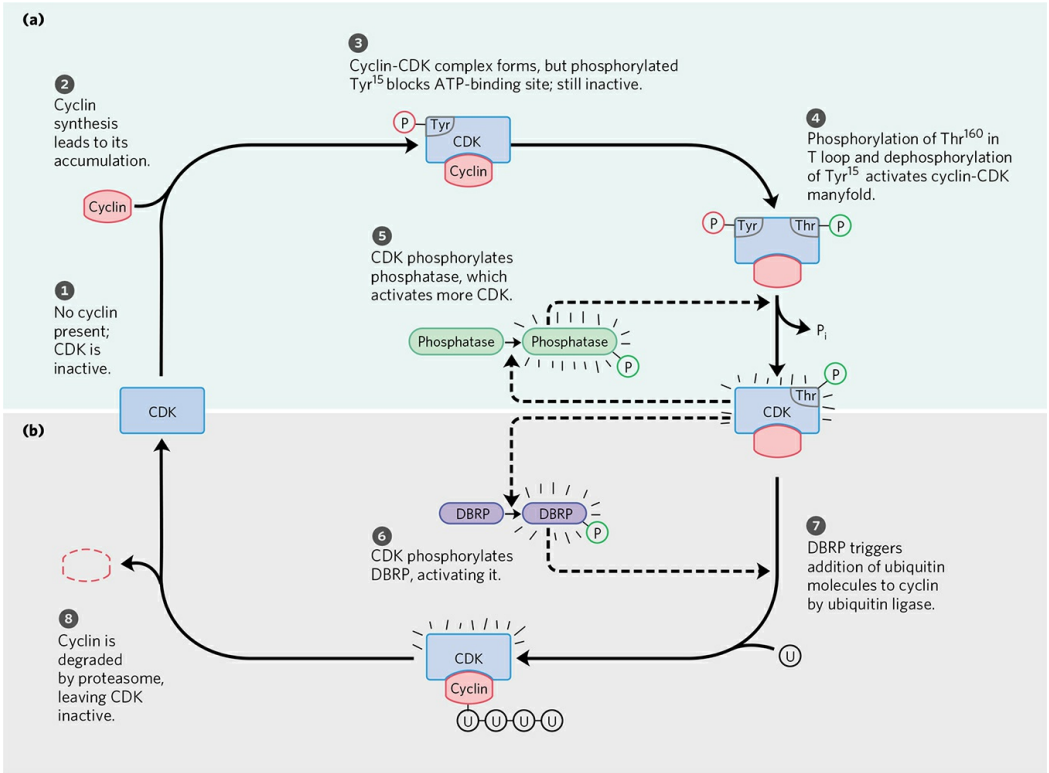


FIGURE 12-35 Regulation of CDK by phosphorylation and proteolysis. (a) As a cell enters mitosis, the M-phase CDK is inactive (step 1). As cyclin is synthesized (step 2), the cyclin-CDK complex forms (step 3). The T loop lies in the substrate-binding site of CDK, and Ⓟ-Tyr^{15} blocks its ATP-binding site, keeping the complex inactive. When Thr^{160} in the T loop is phosphorylated, the loop moves

out of the substrate-binding site, and when Tyr^{15} is dephosphorylated, ATP can bind. These two changes activate the cyclin-CDK manyfold (step 4). Further activation is achieved as CDK also phosphorylates and activates the enzyme that dephosphorylates Ⓟ-Tyr^{15} (step 5). **(b)** The active cyclin-CDK complex triggers its own inactivation by phosphorylation of DBRP (destruction box recognizing protein; step 6). DBRP and ubiquitin ligase then attach several molecules of ubiquitin (U) to the cyclin (step 7), targeting it for destruction by proteolytic enzyme complexes called proteasomes (step 8).

Inhibition of CDKs Finally, specific protein inhibitors bind to and inactivate specific CDKs. One such protein is p21, which we discuss below.

These four control mechanisms modulate the activity of specific CDKs that, in turn, control whether a cell will divide, differentiate, become permanently quiescent, or begin a new cycle of division after a period of quiescence. The details of cell cycle regulation, such as the number of different cyclins and kinases and the combinations in which they act, differ from species to species, but the basic mechanism has been conserved in the evolution of all eukaryotic cells.

CDKs Regulate Cell Division by Phosphorylating Critical Proteins

We have examined how cells maintain close control of CDK activity, but how does the activity of CDKs control the cell cycle? The list of target proteins that CDKs are known to act upon continues to grow, and much remains to be learned. But we can see a general pattern behind CDK regulation by inspecting the effect of CDKs on the structures of lamin and myosin and on the activity of retinoblastoma protein.

The structure of the nuclear envelope is maintained in part by highly organized meshworks of intermediate filaments composed of the protein **lamin**. Breakdown of the nuclear envelope before segregation of the sister chromatids in mitosis is partly due to the phosphorylation of lamin by a CDK, which causes lamin filaments to depolymerize.

A second kinase target is the ATP-driven contractile machinery (actin and myosin) that pinches a dividing cell into two equal parts during cytokinesis. After the division, a CDK phosphorylates a small regulatory subunit of myosin, causing dissociation of myosin from actin filaments and inactivating the contractile machinery. Subsequent dephosphorylation

allows reassembly of the contractile apparatus for the next round of cytokinesis.

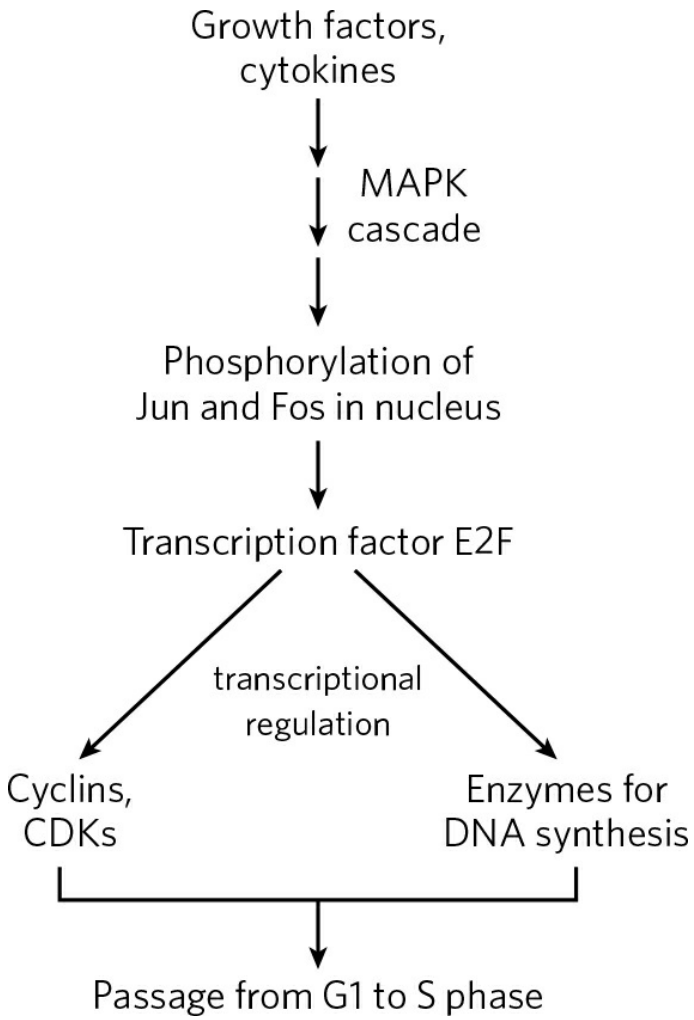


FIGURE 12-36 Regulation of cell division by growth factors. The path from growth factors to cell division leads through the enzyme cascade that activates MAPK, the phosphorylation of the nuclear transcription factors Jun and Fos, and the activity of the transcription factor E2F, which promotes synthesis of several enzymes essential for DNA synthesis.

A third and very important CDK substrate is the **retinoblastoma protein, pRb**; when DNA damage is detected, this protein participates in a mechanism that arrests cell division in G1 **(Fig. 12-37)**. Named for the retinal tumor cell line in which it was discovered, pRb functions in most, perhaps all, cell types to regulate cell division in response to a variety of stimuli. Unphosphorylated pRb binds the transcription factor E2F; while bound to pRb, E2F cannot promote transcription of a group of genes necessary for DNA synthesis (the genes for DNA polymerase α, ribonucleotide reductase, and other proteins; see Chapter 25). In this state,

the cell cycle cannot proceed from the G1 to the S phase, the step that commits a cell to mitosis and cell division. The pRb-E2F blocking mechanism is relieved when pRb is phosphorylated by cyclin E–CDK2, which occurs in response to a signal for cell division to proceed.

When the protein kinases ATM and ATR detect damage to DNA (signaled by the presence of the protein MRN at a double-strand break site), they phosphorylate p53, activating it to serve as a transcription factor that stimulates the synthesis of the protein p21 (Fig. 12-37). This protein inhibits the protein kinase activity of cyclin E–CDK2. In the presence of p21, pRb remains unphosphorylated and bound to E2F, blocking the activity of this transcription factor, and the cell cycle is arrested in G1. This gives the cell time to repair its DNA before entering the S phase, thereby avoiding the potentially disastrous transfer of a defective genome to one or both daughter cells. When the damage is too severe to allow effective repair, this same machinery triggers apoptosis (described below), a process that leads to the death of the cell, preventing the possible development of a cancer.

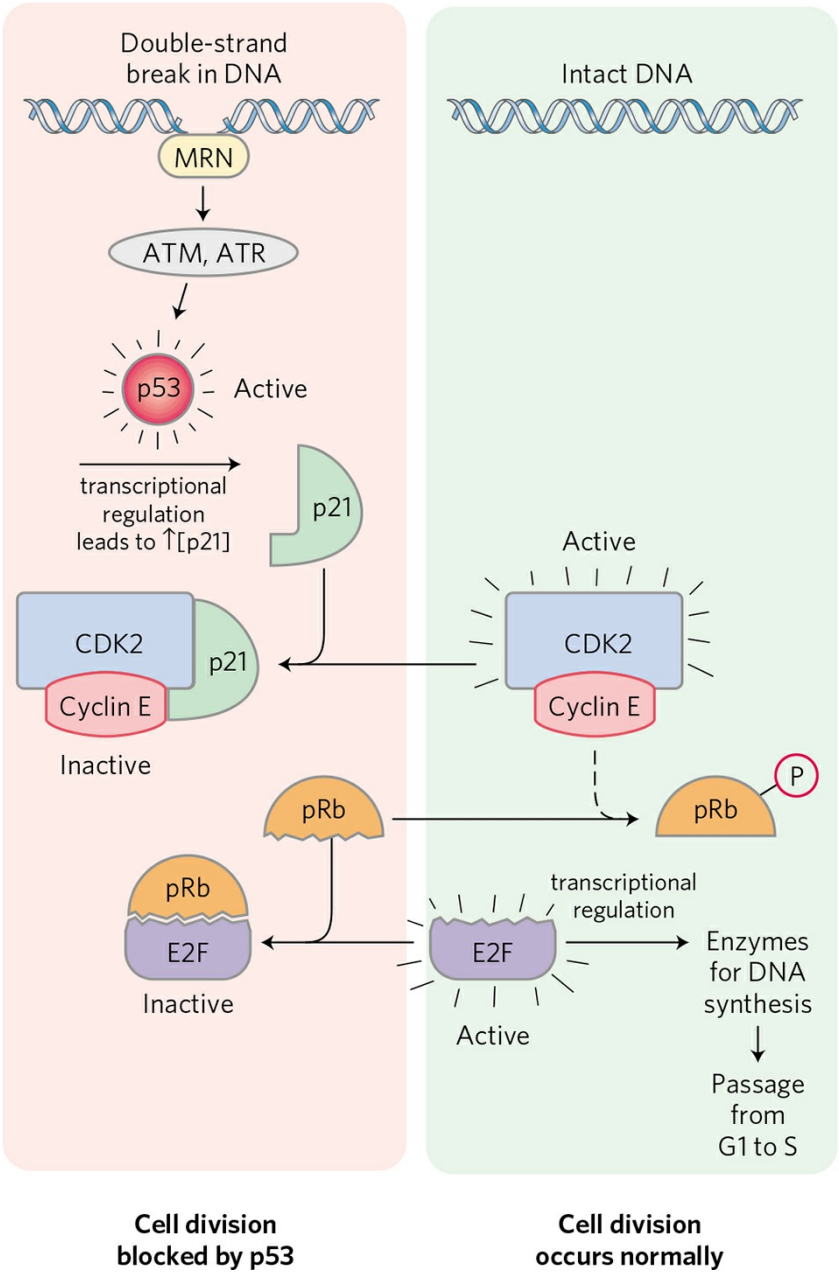


FIGURE 12-37 Regulation of passage from G1 to S by phosphorylation of pRb. Transcription factor E2F promotes transcription of genes for certain enzymes essential to DNA synthesis. The retinoblastoma protein, pRb, can bind E2F (lower left), inactivating it and preventing transcription of these genes. Phosphorylation of pRb by CDK2 prevents it from binding and inactivating E2F, and the genes are transcribed, allowing cell division. Damage to the cell's DNA (upper left) triggers a series of events that inactivate CDK2, blocking cell division. When the protein MRN detects damage to the DNA, it activates two protein kinases, ATM and ATR, and they phosphorylate and activate the transcription factor p53. Active p53 promotes the synthesis of another protein, p21, an inhibitor of CDK2. Inhibition of CDK2 stops the phosphorylation of pRb, which therefore continues to

bind and inhibit E2F. With E2F inactivated, genes essential to cell division are not transcribed and cell division is blocked. When DNA has been repaired, this inhibition is released, and the cell divides.

SUMMARY 12.10 Regulation of the Cell Cycle by Protein Kinases

- Progression through the cell cycle is regulated by the cyclin-dependent protein kinases (CDKs), which act at specific points in the cycle, phosphorylating key proteins and modulating their activities. The catalytic subunit of CDKs is inactive unless associated with the regulatory cyclin subunit.
- The activity of a cyclin-CDK complex changes during the cell cycle through differential synthesis of CDKs, specific degradation of the cyclin, phosphorylation and dephosphorylation of critical residues in CDKs, and binding of inhibitory proteins to specific cyclin-CDKs.
- Among the targets phosphorylated by cyclin-CDKs are proteins of the nuclear envelope and proteins required for cytokinesis and DNA repair.

12.11 Oncogenes, Tumor Suppressor Genes, and Programmed Cell Death

Tumors and cancer are the result of uncontrolled cell division. Normally, cell division is regulated by a family of extracellular growth factors, proteins that cause resting cells to divide and, in some cases, differentiate. The result is a precise balance between the formation of new cells and cell destruction. Regulation of cell division ensures that skin cells are replaced every few weeks and white blood cells are replaced every few days. When this balance is disturbed by defects in regulatory proteins, the result is sometimes the formation of a clone of cells that divide repeatedly and without regulation (a tumor) until their presence interferes with the function of normal tissues—cancer. The direct cause is almost always a genetic defect in one or more of the proteins that regulate cell division. In some cases, a defective gene is inherited from one parent; in other cases, the mutation occurs when a toxic compound from the environment (a mutagen or carcinogen) or high-energy radiation interacts with the DNA of a single cell to damage it and introduce a mutation. In most cases there is both an inherited and an environmental contribution, and in most cases, more than one mutation is required to cause completely unregulated division and full-blown cancer.

Oncogenes Are Mutant Forms of the Genes for Proteins That Regulate the Cell Cycle

Oncogenes are mutated versions of genes encoding signaling proteins involved in cell cycle regulation. Oncogenes were originally discovered in tumor-causing viruses, then later found to be derived from genes in animal host cells, **proto-oncogenes**, which encode growth-regulating proteins. During a viral infection, the host DNA sequence of a proto-oncogene is sometimes copied into the viral genome, where it proliferates with the virus. In subsequent viral infection cycles, the proto-oncogenes can become defective by truncation or mutation. Viruses, unlike animal cells, do not have effective mechanisms for correcting mistakes during DNA replication, so they accumulate mutations rapidly. When a virus carrying an oncogene infects a new host cell, the viral DNA (and oncogene) can be incorporated into the host cell's DNA, where it can now interfere with the regulation of cell division in the host cell. In an alternative, nonviral mechanism, a single cell in a tissue

exposed to carcinogens may suffer DNA damage that renders one of its regulatory proteins defective, with the same effect as the viral oncogenic mechanism: failed regulation of cell division.

The mutations that produce oncogenes are genetically dominant; if either of a pair of chromosomes contains a defective gene, that gene product sends the signal "divide," and a tumor may result. The oncogenic defect can be in any of the proteins involved in communicating the "divide" signal. Oncogenes discovered thus far include those that encode secreted proteins that act as signaling molecules, growth factors, transmembrane proteins (receptors), cytoplasmic proteins (G proteins and protein kinases), and the nuclear transcription factors that control the expression of genes essential for cell division (Jun, Fos).

Some oncogenes encode surface receptors with defective or missing signal-binding sites, such that their intrinsic Tyr kinase activity is unregulated. For example, the oncoprotein ErbB is essentially identical to the normal receptor for epidermal growth factor, except that ErbB lacks the amino-terminal domain that normally binds EGF **(Fig. 12-38)** and is therefore locked in its activated conformation. As a result, the mutant ErbB protein sends the "divide" signal whether EGF is present or not. Mutations in *erbB2*, the gene for a receptor Tyr kinase related to ErbB, are commonly associated with cancers of the glandular epithelium in breast, stomach, and ovary. (For an explanation of the use of abbreviations in naming genes and their products, see Chapter 25.)

The prominent role played by protein kinases in signaling processes related to normal and abnormal cell division has made these enzymes a prime target in the development of drugs for the treatment of cancer (Box 12-4). Mutant forms of the G protein Ras are common in tumor cells. The *ras* oncogene encodes a protein with normal GTP binding but no GTPase activity. The mutant Ras protein is therefore always in its activated (GTP-bound) form, regardless of the signals arriving through normal receptors. The result can be unregulated growth. Mutations in *ras* are associated with 30% to 50% of lung and colon carcinomas and more than 90% of pancreatic carcinomas. ■

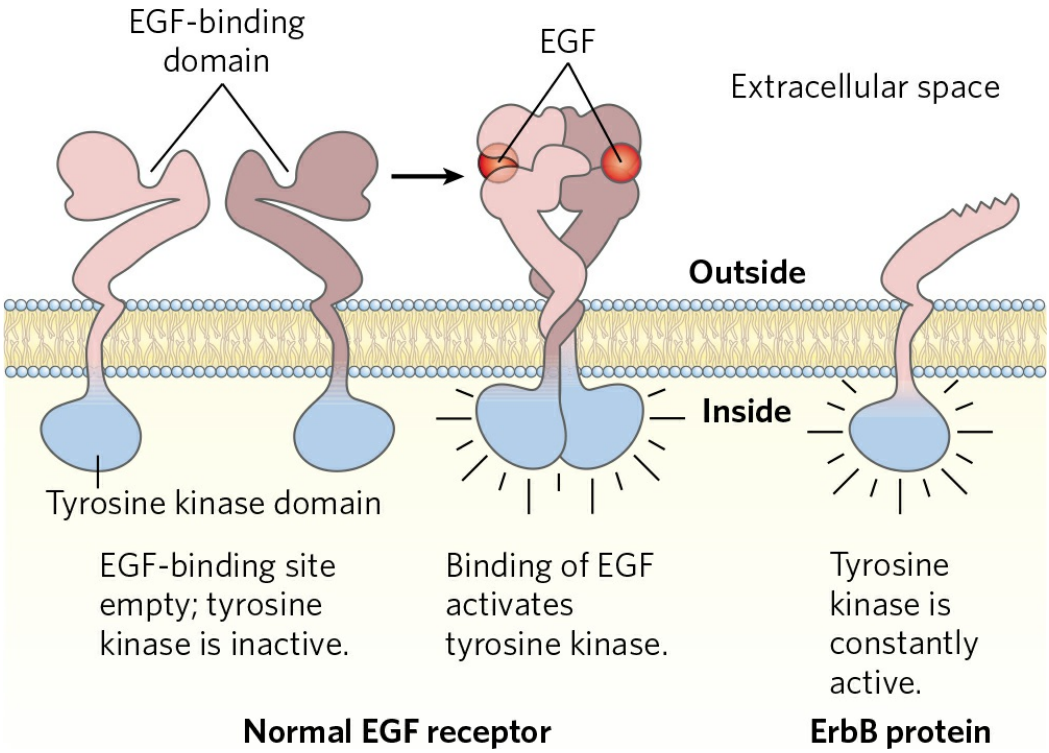


FIGURE 12-38 Oncogene-encoded defective EGF receptor. The product of the *erbB2* oncogene (the ErbB protein) is a truncated version of the normal receptor for epidermal growth factor (EGF). Its intracellular domain has the structure normally induced by EGF binding, but the protein lacks the extracellular binding site for EGF. Unregulated by EGF, ErbB continuously signals cell division.

BOX 12-4 MEDICINE Development of Protein Kinase Inhibitors for Cancer Treatment

When a single cell divides without any regulatory limitation, it eventually gives rise to a clone of cells so large that it interferes with normal physiological functions (Fig. 1). This is cancer, a leading cause of death in the developed world, and increasingly so in the developing world. In all types of cancer, the normal regulation of cell division has become dysfunctional due to defects in one or more genes. For example, genes encoding proteins that normally send intermittent signals for cell division become oncogenes, producing constitutively active signaling proteins, or genes encoding proteins that normally restrain cell division (tumor suppressor genes) mutate to produce

proteins that lack this braking function. In many tumors, both kinds of mutation have occurred.

Many oncogenes and tumor suppressor genes encode protein kinases or proteins that act in pathways upstream from protein kinases. It is therefore reasonable to hope that specific inhibitors of protein kinases could prove valuable in the treatment of cancer. For example, a mutant form of the EGF receptor is a constantly active receptor Tyr kinase (RTK), signaling cell division whether EGF is present or not (see Fig. 12-38). In about 30% of all women with invasive breast cancer, a mutation in the gene for the receptor HER2/neu yields an RTK with activity increased up to 100-fold. Another RTK, **vascular endothelial growth factor receptor (VEGFR)**, must be activated for the formation of new blood vessels (angiogenesis) to provide a solid tumor with its own blood supply, and inhibition of VEGFR might starve a tumor of essential nutrients. Nonreceptor Tyr kinases can also mutate, resulting in constant signaling and unregulated cell division. For example, the oncogene *Abl* (from the *Ab*elson *l*eukemia virus) is associated with acute myeloid leukemia, a relatively rare blood disease (~5,000 cases a year in the United States). Another group of oncogenes encode unregulated cyclin-dependent protein kinases. In each of these cases, specific protein kinase inhibitors might be valuable chemotherapeutic agents in the treatment of disease. Not surprisingly, huge efforts are under way to develop such inhibitors. How should one approach this challenge?

FIGURE 1 Unregulated division of a single cell in the colon led to a primary cancer that metastasized to the liver. Secondary cancers are seen as white patches in this liver obtained at autopsy.
[Source: CNRI/Science Source.]

Protein kinases of all types show striking conservation of structure at the active site. All share with the prototypical PKA structure the features shown in Figure 2: two lobes that enclose the active site, with a P loop that helps to align and bind the phosphoryl groups of ATP, an activation loop that moves to open the active site to the protein substrate, and a C helix that changes position as the enzyme is activated, bringing the residues in the substrate-binding cleft into their binding positions.

The simplest protein kinase inhibitors are ATP analogs that occupy the ATP-binding site but cannot serve as phosphoryl group donors. Many such compounds are known, but their clinical usefulness is limited by their lack of selectivity—they inhibit virtually all protein kinases and would produce unacceptable side effects. More selectivity is seen with compounds that fill part of the ATP-binding site but also interact outside this site with parts of the protein unique to the target protein kinase. A third possible strategy is based on the fact that although the active conformations of all protein kinases are similar, their inactive conformations are not. Drugs that target the inactive conformation of a specific protein kinase and prevent its conversion to the active form may have a higher specificity of action. A fourth approach employs the great specificity of antibodies. For example, monoclonal antibodies (p. 177) that bind the extracellular portions of specific RTKs could eliminate the receptors' kinase activity by preventing dimerization or by causing their removal from the cell surface. In some cases, an antibody selectively binding to the surface of cancer cells could cause the immune system to attack those cells.

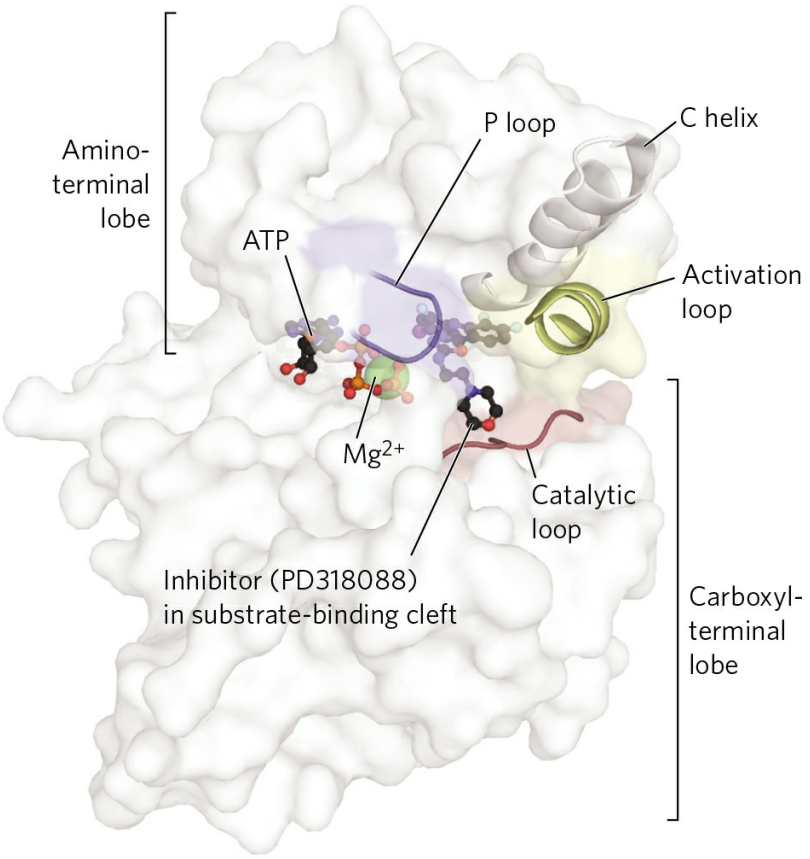


FIGURE 2 Conserved features of the active site of protein kinases. The amino-terminal and carboxyl-terminal lobes surround the active site of the enzyme, near the catalytic loop and the site where ATP binds. The activation loop of this and many other kinases undergoes phosphorylation, then moves away from the active site to expose the substrate-binding cleft, which in this image is occupied by a specific inhibitor of this enzyme, PD318088. The P loop is essential in the binding of ATP, and the C helix must also be correctly aligned for ATP binding and kinase activity.
[Source: PDB ID 1S9I, J. F. Ohren et al., *Nature Struct. Mol. Biol.* 11:1192, 2004.]

The search for drugs active against specific protein kinases has yielded encouraging results. For example, imatinib mesylate (Gleevec; Fig. 3a), one of the small-molecule inhibitors, has proved nearly 100% effective in bringing about remission in patients with early-stage chronic myeloid leukemia. Erlotinib (Tarceva; Fig. 3b), which targets EGFR, is effective against advanced non-small-cell lung cancer (NSCLC). Because many cell-division signaling systems involve more than one protein kinase, inhibitors that act on several protein kinases may be useful in the treatment of cancer. Sunitinib (Sutent) and sorafenib (Nexavar) target several protein kinases, including VEGFR

and PDGFR. These two drugs are in clinical use for patients with gastrointestinal stromal tumors and advanced renal cell carcinoma, respectively. Trastuzumab (Herceptin), cetuximab (Erbitux), and bevacizumab (Avastin) are monoclonal antibodies that target HER2/neu, EGFR, and VEGFR, respectively; all three drugs are in clinical use for certain types of cancer. Detailed knowledge of the structure around the ATP-binding site makes it possible to design drugs that inhibit a *specific* protein kinase by (1) blocking the critical ATP-binding site, while (2) interacting with residues around that site that are *unique* to that particular protein kinase.

At least a hundred more compounds are in preclinical trials. Among the drugs being evaluated are some obtained from natural sources and some produced by synthetic chemistry. Indirubin is a component of a Chinese herbal preparation traditionally used to treat certain leukemias; it inhibits CDK2 and CDK5. Roscovatine (Fig. 3d), a substituted adenine, has a benzyl ring that makes it highly specific as an inhibitor of CDK2. With several hundred potential anticancer drugs heading toward clinical testing, it is realistic to hope that some will prove more effective or more target-specific than those now in use.

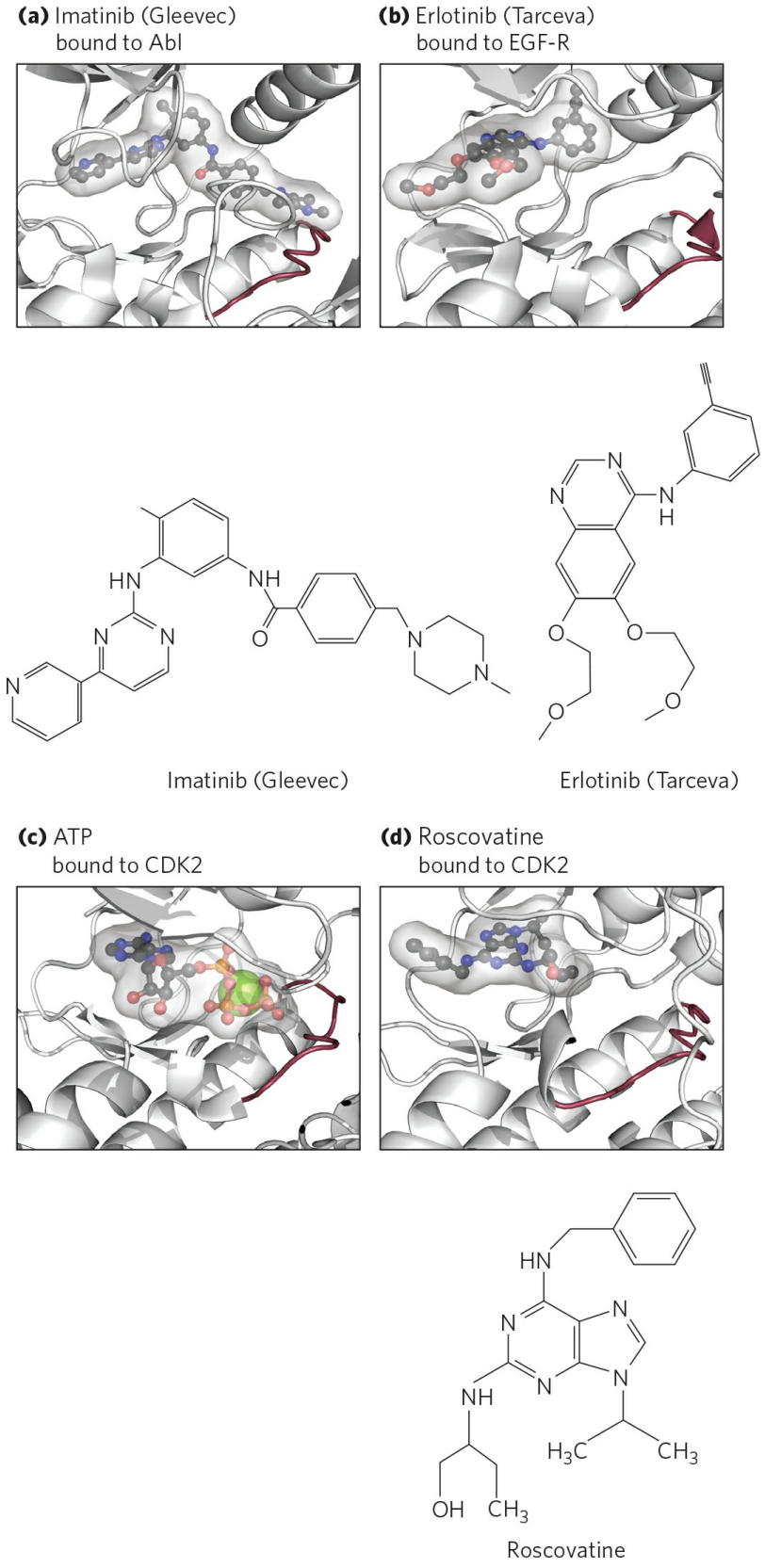


FIGURE 3 Some protein kinase inhibitors now in clinical trials or clinical use, showing their binding to the target protein. **(a)** Imatinib binds to the Abl kinase (an oncogene product) active site; it occupies both the ATP-binding site and a region adjacent to that site. **(b)**

Erlotinib binds to the active site of EGFR. **(c)**, **(d)** Roscovatine is an inhibitor of the cyclin-dependent kinases CDK2, CDK7, and CDK9; shown here are normal Mg^{2+}-ATP binding at the active site (c) and roscovatine binding (d), which prevents the binding of ATP. [Sources: (a) PDB ID 1IEP, B. Nagar et al., *Cancer Res.* 62:4236, 2002. (b) PDB ID 1M17, J. Stamos et al., *J. Biol. Chem.* 277:46,265, 2002. (c) PDB ID 1S9I, J. F. Ohren et al., *Nature Struct. Mol. Biol.* 11:1192, 2004. (d) PDB ID 2A4L, W. F. De Azevedo et al., *Eur. J. Biochem.* 243:518, 1997.]

Defects in Certain Genes Remove Normal Restraints on Cell Division

Tumor suppressor genes encode proteins that normally restrain cell division. Mutation in one or more of these genes can lead to tumor formation. Unregulated growth due to defective tumor suppressor genes, unlike that due to oncogenes, is genetically recessive; tumors form only if *both* chromosomes of a pair contain a defective gene. This is because the function of these genes is to prevent cell division, and if either copy of the gene is normal, it will produce a normal protein and normal inhibition of division. In a person who inherits one correct copy and one defective copy, every cell begins with one defective copy of the gene. If any one of the individual's 10^{12} somatic cells undergoes mutation in the one good copy, a tumor may grow from that doubly mutant cell. Mutations in both copies of the genes for pRb, p53, or p21 yield cells in which the normal restraint on cell division is lost and a tumor forms.

Retinoblastoma occurs in children and causes blindness if not surgically treated. The cells of a retinoblastoma have two defective versions of the *Rb* gene (two defective alleles). Very young children who develop retinoblastoma commonly have multiple tumors in both eyes. These children have inherited one defective copy of the *Rb* gene, which is present in every cell; each tumor is derived from a single retinal cell that has undergone a mutation in its one good copy of the *Rb* gene. (A fetus with two mutant alleles in every cell is nonviable.) People with retinoblastoma who survive childhood also have a high incidence of cancers of the lung, prostate, and breast later in life.

A far less likely event is that a person born with two good copies of the *Rb* gene will have independent mutations in both copies in the *same* cell. Some individuals do develop retinoblastomas later in childhood, usually

with only one tumor in one eye. These individuals, presumably, were born with two good copies (alleles) of *Rb* in every cell, but both *Rb* alleles in a single retinal cell have undergone mutation, leading to a tumor. After about age three, retinal cells stop dividing, and retinoblastomas at later ages are quite rare.

Stability genes (also called caretaker genes) encode proteins that function in the repair of major genetic defects that result from aberrant DNA replication, ionizing radiation, or environmental carcinogens. Mutations in these genes lead to a high frequency of unrepaired damage (mutations) in other genes, including proto-oncogenes and tumor suppressor genes, and thus to cancer. Among the stability genes are *ATM* (see Fig. 12-37); the *XP* gene family, in which mutations lead to xeroderma pigmentosum; and the *BRCA1* genes associated with some types of breast cancer (see Box 25-1). Mutations in the gene for p53 also cause tumors; in more than 90% of human cutaneous squamous cell carcinomas (skin cancers) and in about 50% of all other human cancers, *p53* is defective. Those very rare individuals who *inherit* one defective copy of *p53* commonly have the Li-Fraumeni cancer syndrome, with multiple cancers (of the breast, brain, bone, blood, lung, and skin) occurring at high frequency and at an early age. The explanation for multiple tumors in this case is the same as that for *Rb* mutations: an individual born with one defective copy of *p53* in every somatic cell is likely to suffer a second *p53* mutation in more than one cell during his or her lifetime.

In summary, then, three classes of defects can contribute to the development of cancer: (1) oncogenes, in which the defect is the equivalent of a car's accelerator pedal being stuck down, with the engine racing; (2) mutated tumor suppressor genes, in which the defect leads to the equivalent of brake failure; and (3) mutated stability genes, with the defect leading to unrepaired damage to the cell's replication machinery—the equivalent of an unskilled car mechanic.

Mutations in oncogenes and tumor suppressor genes do not have an all-or-none effect. In some cancers, perhaps in all, the progression from a normal cell to a malignant tumor requires an accumulation of mutations (sometimes over several decades), none of which, alone, is responsible for the end effect. For example, the development of colorectal cancer has several recognizable stages, each associated with a mutation **(Fig. 12-39)**. If an epithelial cell in the colon undergoes mutation of both copies of the tumor suppressor gene *APC* (adenomatous polyposis coli), it begins to divide faster than normal and produces a clone of itself, a benign polyp

(early adenoma). For reasons not yet known, the *APC* mutation results in chromosomal instability, and whole regions of a chromosome are lost or rearranged during cell division. This instability can lead to another mutation, commonly in *ras*, that converts the clone into an intermediate adenoma. A third mutation (often in the tumor suppressor gene *DCC*) leads to a late adenoma. Only when both copies of *p53* become defective does this cell mass become a carcinoma—a malignant, life-threatening tumor. The full sequence therefore requires at least seven genetic "hits": two on each of three tumor suppressor genes (*APC*, *DCC*, and *p53*) and one on the proto-oncogene *ras*. There are probably several other routes to colorectal cancer as well, but the principle that full malignancy results only from multiple mutations is likely to hold true for all of them. Because mutations accumulate over time, the chances of developing full-blown metastatic cancer rise with age (Fig. 12-39).

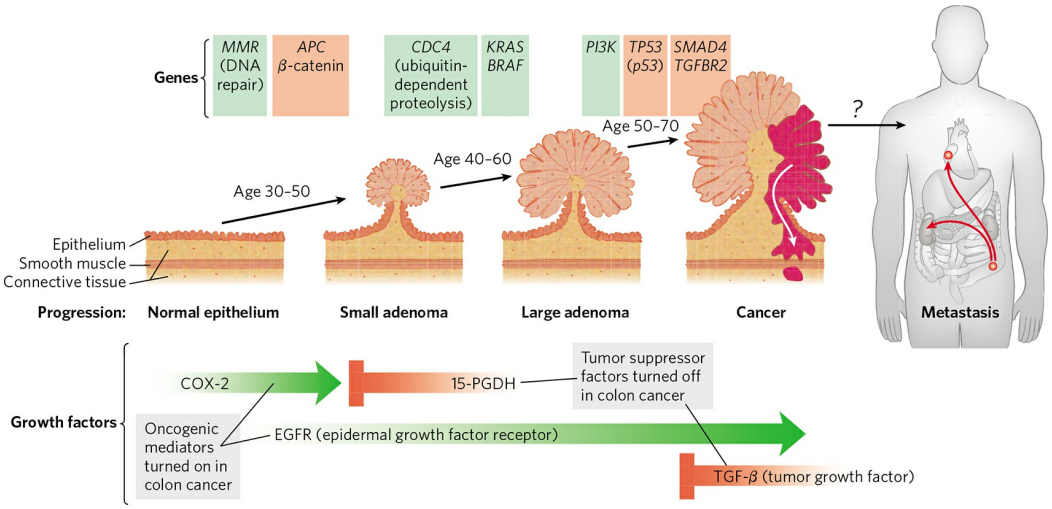


FIGURE 12-39 Multistep transition from normal epithelial cell to colorectal cancer. Serial mutations in oncogenes (green) or tumor suppressor genes (red) lead to progressively less control of cell division, until finally an active tumor forms, which can sometimes metastasize (spread from the initial site to other regions of the body). Mutation of the *MMR* gene leads to defective DNA repair and consequently to a higher rate of mutation. Mutations in both copies of the tumor suppressor gene *APC* lead to benign clusters of epithelial cells that multiply too rapidly (early adenoma). The *CDC4* oncogene results in defective ubiquitination, which is essential to the regulation of cyclin-dependent kinases (see Fig. 12-35). The oncogenes *KRAS* and *BRAF* encode Ras and Raf proteins (see Fig. 12-19), and this further disruption of signaling leads to the formation of a large adenoma, which may be detected by colonoscopy as a benign polyp. Oncogenic mutations in the *PI3K* gene, which encodes the enzyme phosphoinositide-3 kinase, or in

PTEN, which regulates the synthesis of this enzyme, lead to a further strengthening of the signal: divide now. When a cell in one of the polyps undergoes further mutations, such as in the tumor suppressor genes *DCC* and *p53* (see Fig. 12-37), increasingly aggressive tumors form. Finally, mutations in other tumor suppressor genes such as *SMAD4* lead to a malignant tumor and sometimes to a metastatic tumor that can spread to other tissues. A second type of mutation that can add to the deleterious effects is one that affects the production or action of growth factors or their receptors (bottom). Mutations in EGFR (epidermal growth factor receptor) or TGF-β (transforming growth factor-β) favor uncontrolled growth, as do mutations in the enzymes that produce certain prostaglandins (COX-2; cyclooxygenase; see Fig. 10-17) or the enzyme 15-PGDH (15-hydroxyprostaglandin dehydrogenase). Most malignant tumors of other tissues probably result from a series of mutations such as this, although not necessarily these particular genes, or in this order.
[Source: Information from S. D. Markowitz and M. M. Bertagnolli, *N. Engl. J. Med.* 361:2449, 2009, Fig. 2.]

When a polyp is detected in the early adenoma stage and the cells containing the first mutations are removed surgically, late adenomas and carcinomas will not develop; hence the importance of early detection. Cells and organisms, too, have their early detection systems. For example, the ATM and ATR proteins described in Section 12.10 can detect DNA damage too extensive to be repaired effectively. They then trigger, through a pathway that includes p53, the process of apoptosis, in which a cell that has become dangerous to the organism kills itself.

The development of fast and inexpensive sequencing methods has opened a new window on the process by which cancer develops. In a typical study of cancers in humans, the sequences of all 20,000 genes were determined in about 3,300 different tumors, and then compared with the gene sequences in noncancerous tissue from the same patient. Almost 300,000 mutations were detected in all. Only a small fraction of these mutations, the **driver mutations**, were the *cause* of unregulated cell division; the vast majority (>99.9%) were "passenger mutations," which occurred randomly and did not confer a selective growth advantage on the tissue in which they occurred. Among the driver mutations were those in about 75 tumor suppressor genes and about 65 oncogenes. These 140 driver mutations fell in three general categories: those that affect cell survival (in genes encoding Ras, PI3K, MAPK, for example), those that affect cells' ability to maintain an intact genome (ATM, ATR), and those that affect cell fate, causing cells to divide, differentiate, or become

quiescent (APC is one example). A relatively small number of mutations were very common in multiple types of cancer, in the genes for Ras, p53, and pRb, for example. ■

Apoptosis Is Programmed Cell Suicide

Many cells can precisely control the time of their own death by the process of **programmed cell death**, or **apoptosis** (pronounced app′-a-toe′-sis; from the Greek for "dropping off," as in leaves dropping in the fall). One trigger for apoptosis is irreparable damage to DNA. Programmed cell death also occurs during the normal development of an embryo, when some cells must die to give a tissue or organ its final shape. Carving fingers from stubby limb buds requires the precisely timed death of cells between developing finger bones. During development of the nematode *C. elegans* from a fertilized egg, exactly 131 cells (of a total of 1,090 somatic cells in the embryo) must undergo programmed death in order to construct the adult body.

Apoptosis also has roles in processes other than development. If a developing antibody-producing cell generates antibodies against a protein or glycoprotein normally present in the body, that cell undergoes programmed death in the thymus gland—an essential mechanism for eliminating anti-self antibodies (the cause of many autoimmune diseases). The monthly sloughing of cells of the uterine wall (menstruation) is another case of apoptosis mediating normal cell death. The dropping of leaves in the fall is the result of apoptosis in specific cells of the stem. Sometimes cell suicide is not programmed but occurs in response to biological circumstances that threaten the rest of the organism. For example, a virus-infected cell that dies before completion of the infection cycle prevents spread of the virus to nearby cells. Severe stresses such as heat, hyperosmolarity, UV light, and gamma irradiation also trigger cell suicide; presumably the organism is better off with any aberrant, potentially mutated cells dead.

The regulatory mechanisms that trigger apoptosis involve some of the same proteins that regulate the cell cycle. The signal for suicide often comes from outside, through a surface receptor. Tumor necrosis factor (TNF), produced by cells of the immune system, interacts with cells through specific TNF receptors. These receptors have TNF-binding sites on the outer face of the plasma membrane and a "death domain" (~80 amino acid residues) that carries the self-destruct signal through the membrane to cytosolic proteins such as TRADD (TNF receptor–associated death domain) **(Fig. 12-40)**.

When caspase 8, an “initiator” caspase, is activated by an apoptotic signal carried through TRADD, it further self-activates by cleaving its own proenzyme form. Mitochondria are one target of active caspase 8. The protease causes the release of certain proteins contained between the inner and outer mitochondrial membranes: cytochrome *c* and several “effector” caspases (see Fig. 19-39). Cytochrome *c* binds to the proenzyme form of the effector enzyme caspase 9 and stimulates its proteolytic activation. The activated caspase 9, in turn, catalyzes wholesale destruction of cellular proteins—a major cause of apoptotic cell death. One specific target of caspase action is a caspase-activated deoxyribonuclease.

In apoptosis, the monomeric products of protein and DNA degradation (amino acids and nucleotides) are released in a controlled process that allows them to be taken up and reused by neighboring cells. Apoptosis thus allows the organism to eliminate a cell that is unneeded or potentially dangerous without wasting its components.

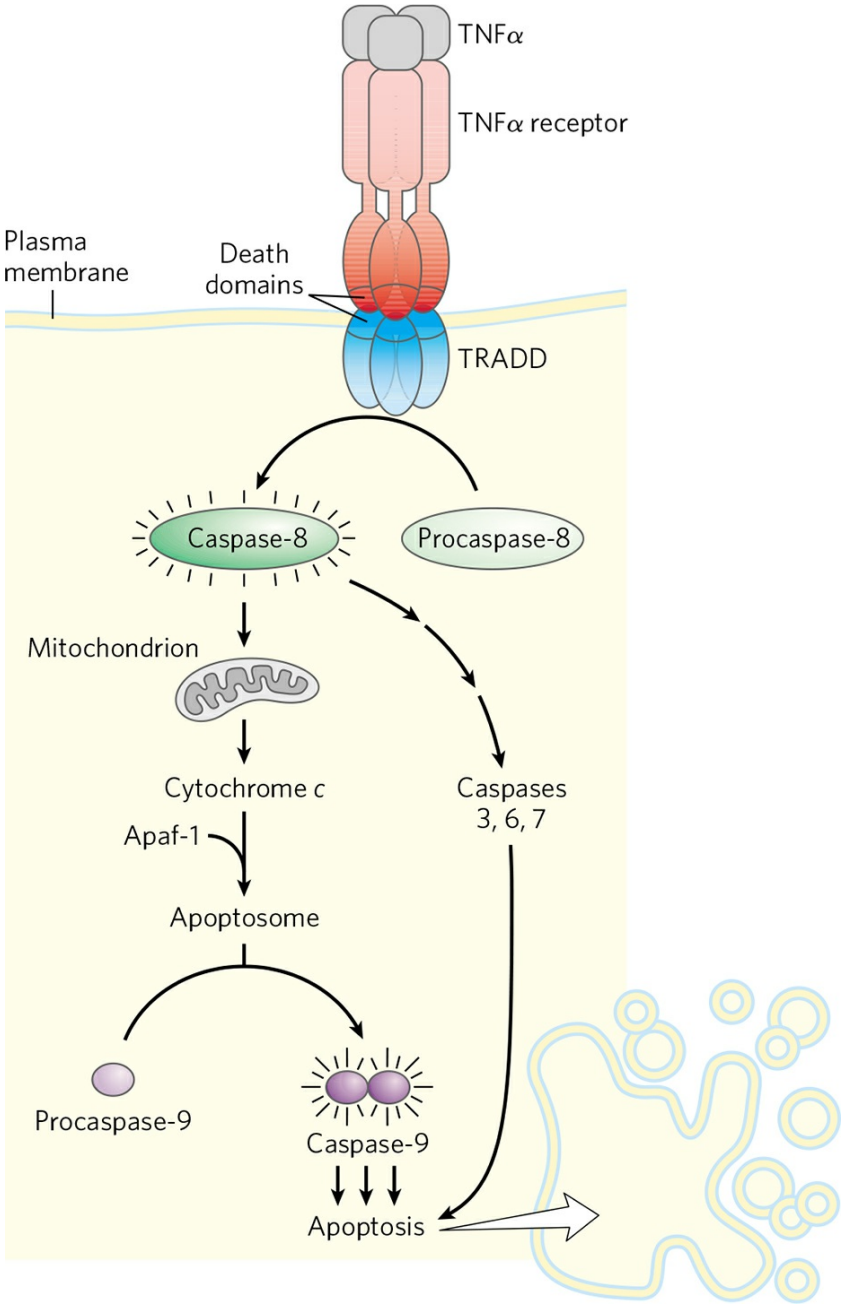


FIGURE 12-40 Initial events of apoptosis. An apoptosis-triggering signal from outside the cell (TNFa) binds to its specific receptor in the plasma membrane. The occupied receptor interacts with the cytosolic protein TRADD through "death domains" (80-residue domains on both TNFa receptor and TRADD), activating TRADD. Activated TRADD initiates a proteolytic cascade that leads to apoptosis: TRADD activates caspase-8, which acts to release cytochrome c from mitochondria, which, in concert with protein Apaf-1, activates caspase-9, triggering apoptosis (see Fig. 19-39).

SUMMARY 12.11 Oncogenes, Tumor Suppressor Genes, and Programmed Cell Death

- Oncogenes encode defective signaling proteins. By continually giving the signal for cell division, they lead to tumor formation. Oncogenes are genetically dominant and may encode defective growth factors, receptors, G proteins, protein kinases, or nuclear regulators of transcription.
- Tumor suppressor genes encode regulatory proteins that normally inhibit cell division; mutations in these genes are genetically recessive but can lead to tumor formation.
- Cancer is generally the result of an accumulation of mutations in oncogenes and tumor suppressor genes.
- When stability genes, which encode proteins necessary for the repair of genetic damage, are mutated, other mutations go unrepaired, including mutations in proto-oncogenes and tumor suppressor genes that can lead to cancer.
- Apoptosis is programmed and controlled cell death that functions during normal development and adulthood to get rid of unnecessary, damaged, or infected cells. Apoptosis can be triggered by extracellular signals such as TNF, acting through plasma membrane receptors.

Key Terms

Terms in bold are defined in the glossary.

signal transduction
specificity
cooperativity
amplification
enzyme cascade
modularity
scaffold proteins
desensitization
integration
response localization
G protein–coupled receptors (GPCRs)
guanosine nucleotide–binding proteins
G proteins
second messenger
agonist
antagonist
β-adrenergic receptors
heptahelical receptors
stimulatory G protein (G_s)
adenylyl cyclase
cAMP-dependent protein kinase (protein kinase A; PKA)
P loop
GTPase activator protein (GAP)
regulator of G protein signaling (RGS)
guanosine nucleotide–exchange factor (GEF)
consensus sequence
β-arrestin (*β*arr; arrestin 2)
G protein–coupled receptor kinases (GRKs)
cAMP response element binding protein (CREB)
inhibitory G protein (G_i)
adaptor proteins
AKAPs (A kinase anchoring proteins)
phospholipase C (PLC)
inositol 1,4,5-trisphosphate (IP_3)
protein kinase C (PKC)

green fluorescent protein (GFP)
fluorescence resonance energy transfer (FRET)
calmodulin (CaM)
Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases)
rhodopsin
opsin
rhodopsin kinase
receptor potential
gustducin
receptor Tyr kinase (RTK)
autophosphorylation
SH2 domain
Ras
small G proteins
MAPKs
guanosine 3′, 5′-cyclic monophosphate (cyclic GMP; cGMP)
cGMP-dependent protein kinase (protein kinase G; PKG)
atrial natriuretic factor (ANF)
NO synthase
PTB domains
voltage-gated ion channels
ionotropic
metabotropic
hormone response element (HRE)
two-component signaling systems
receptor histidine kinase
response regulator
cyclin
cyclin-dependent protein kinase (CDK)
ubiquitin
proteasome
growth factors
retinoblastoma protein (pRb)
oncogene
proto-oncogene
tumor suppressor gene
programmed cell death
apoptosis

Problems

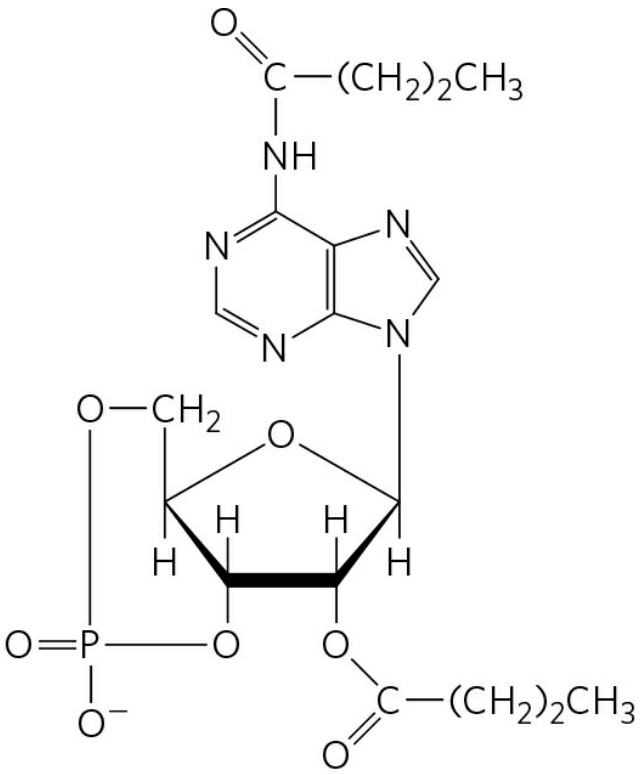
1. Hormone Experiments in Cell-Free Systems In the 1950s, Earl W. Sutherland, Jr., and his colleagues carried out pioneering experiments to elucidate the mechanism of action of epinephrine and glucagon. Given what you have learned in this chapter about hormone action, interpret each of the experiments described below. Identify substance X and indicate the significance of the results.

(a) Addition of epinephrine to a homogenate of normal liver resulted in an increase in the activity of glycogen phosphorylase. However, when the homogenate was first centrifuged at a high speed and epinephrine or glucagon was added to the clear supernatant fraction that contains phosphorylase, no increase in the phosphorylase activity occurred.

(b) When the particulate fraction from the centrifugation in (a) was treated with epinephrine, substance X was produced. The substance was isolated and purified. Unlike epinephrine, substance X activated glycogen phosphorylase when added to the clear supernatant fraction of the centrifuged homogenate.

(c) Substance X was heat stable; that is, heat treatment did not affect its capacity to activate phosphorylase. (Hint: Would this be the case if substance X were a protein?) Substance X was nearly identical to a compound obtained when pure ATP was treated with barium hydroxide. (Fig. 8-6 will be helpful.)

2. Effect of Dibutyryl cAMP versus cAMP on Intact Cells The physiological effects of epinephrine should in principle be mimicked by addition of cAMP to the target cells. In practice, addition of cAMP to intact target cells elicits only a minimal physiological response. Why? When the structurally related derivative dibutyryl cAMP (shown below) is added to intact cells, the expected physiological response is readily apparent. Explain the basis for the difference in cellular response to these two substances. Dibutyryl cAMP is widely used in studies of cAMP function.



Dibutyryl cAMP
(N^6,$O^{2'}$-Dibutyryl adenosine 3′,5′-cyclic monophosphate)

3. Effect of Cholera Toxin on Adenylyl Cyclase The gram-negative bacterium *Vibrio cholerae* produces a protein, cholera toxin (M_r 90,000), that is responsible for the characteristic symptoms of cholera: extensive loss of body water and Na^+ through continuous, debilitating diarrhea. If body fluids and Na^+ are not replaced, severe dehydration results; untreated, the disease is often fatal. When the cholera toxin gains access to the human intestinal tract, it binds tightly to specific sites in the plasma membrane of the epithelial cells lining the small intestine, causing adenylyl cyclase to undergo prolonged activation (hours or days).

(a) What is the effect of cholera toxin on [cAMP] in the intestinal cells?

(b) Based on the information above, suggest how cAMP normally functions in intestinal epithelial cells.

(c) Suggest a possible treatment for cholera.

4. Mutations in PKA Explain how mutations in the R or C subunit of cAMP-dependent protein kinase (PKA) might lead to (a) a constantly active PKA or (b) a constantly inactive PKA.

5. Therapeutic Effects of Albuterol The respiratory symptoms of asthma result from constriction of the bronchi and bronchioles of the lungs, caused by contraction of the smooth muscle of their walls. This constriction can be reversed by raising [cAMP] in the smooth muscle. Explain the therapeutic effects of albuterol, a β-adrenergic agonist taken (by inhalation) for asthma. Would you

expect this drug to have any side effects? If so, how might one design a better drug that does not have these effects?

6. Termination of Hormonal Signals Signals carried by hormones must eventually be terminated. Describe several different mechanisms for signal termination.

7. Using FRET to Explore Protein-Protein Interactions In Vivo Figure 12-8 shows the interaction between *β*-arrestin and the *β*-adrenergic receptor. How would you use FRET (see Box 12-2) to demonstrate this interaction in living cells? Which proteins would you fuse? Which wavelengths would you use to illuminate the cells, and which wavelengths would you monitor? What would you expect to observe if the interaction occurred? If it did not occur? How might you explain the failure of this approach to demonstrate this interaction?

8. EGTA Injection EGTA (ethylene glycol-bis(*β*-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) is a chelating agent with high affinity and specificity for Ca^{2+}. By microinjecting a cell with an appropriate Ca^{2+}-EGTA solution, an experimenter can prevent cytosolic [Ca^{2+}] from rising above 10^{+7} M. How would EGTA microinjection affect a cell's response to vasopressin (see Table 12-4)? To glucagon?

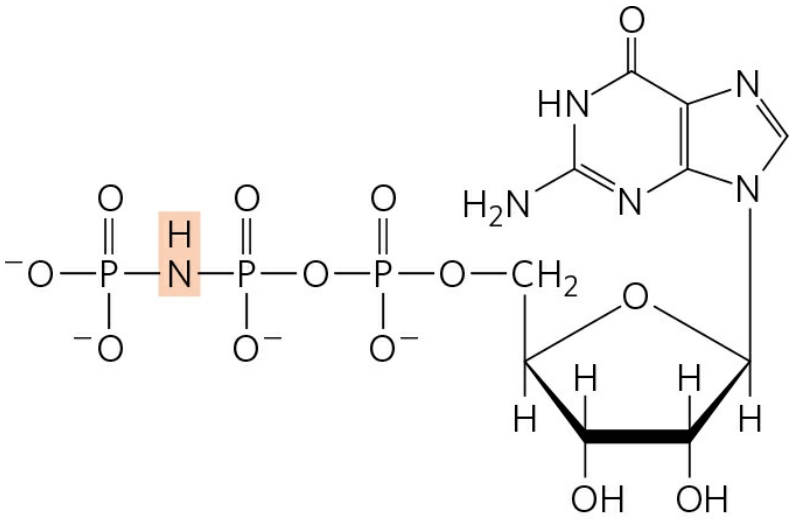
9. Amplification of Hormonal Signals Describe all the sources of amplification in the insulin receptor system.

10. Mutations in *ras* How would a mutation in *ras* that leads to formation of a Ras protein with no GTPase activity affect a cell's response to insulin?

11. Differences among G Proteins Compare the G protein G_s, which acts in transducing the signal from *β*-adrenergic receptors, and the G protein Ras. What properties do they share? How do they differ? What is the functional difference between G_s and G_i?

12. Mechanisms for Regulating Protein Kinases Identify eight general types of protein kinases found in eukaryotic cells, and explain what factor is *directly* responsible for activating each type.

13. Nonhydrolyzable GTP Analogs Many enzymes can hydrolyze GTP between the *β* and *γ* phosphates. The GTP analog *β*,*γ*-imidoguanosine 5′-triphosphate (Gpp(NH)p), shown below, cannot be hydrolyzed between the *β* and *γ* phosphates.



Gpp(NH)p
(β,γ-imidoguanosine 5′-triphosphate)

Predict the effect of microinjection of Gpp(NH)p into a myocyte on the cell's response to β-adrenergic stimulation.

14. Use of Toxin Binding to Purify a Channel Protein α-Bungarotoxin is a powerful neurotoxin found in the venom of a poisonous snake (*Bungarus multicinctus*). It binds with high specificity to the acetylcholine receptor (AChR; an integral membrane protein) and prevents its ion channel from opening. This interaction was used to purify AChR from the electric organ of torpedo fish.

(a) Outline a strategy for using α-bungarotoxin covalently bound to chromatography beads to purify the AChR protein. (Hint: See Fig. 3-17c.)

(b) Outline a strategy for the use of [^{125}I]α-bungarotoxin to purify the AChR protein.

15. Excitation Triggered by Hyperpolarization In most neurons, membrane *depolarization* leads to the opening of voltage-dependent ion channels, generation of an action potential, and, ultimately, an influx of Ca^{2+}, which causes release of neurotransmitter at the axon terminus. Devise a cellular strategy by which *hyperpolarization* in rod cells could produce excitation of the visual pathway and passage of visual signals to the brain. (Hint: The neuronal signaling pathway in higher organisms consists of a *series* of neurons that relay information to the brain. The signal released by one neuron can be either excitatory or inhibitory to the following, postsynaptic neuron.)

16. Visual Desensitization Oguchi disease is an inherited form of night blindness. Affected individuals are slow to recover vision after a flash of bright light against a dark background, such as the headlights of a car on the freeway. Suggest what the molecular defect(s) might be in Oguchi disease. Explain in molecular terms how this defect would account for night blindness.

17. Effect of a Permeant cGMP Analog on Rod Cells An analog of cGMP, 8-Br-cGMP, will permeate cellular membranes, is only slowly degraded by a rod cell's PDE activity, and is as effective as cGMP in opening the gated channel in the cell's outer segment. If you suspended rod cells in a buffer containing a relatively high [8-Br-cGMP], then illuminated the cells while measuring their membrane potential, what would you observe?

18. Hot and Cool Taste Sensations The sensations of heat and cold are transduced by a group of temperature-gated cation channels. For example, TRPV1, TRPV3, and TRPM8 are usually closed, but open under the following conditions: TRPV1 at ≥43 °C; TRPV3 at ≥33 °C; and TRPM8 at <25 °C. These channel proteins are expressed in sensory neurons known to be responsible for temperature sensation.

(a) Propose a reasonable model to explain how exposing a sensory neuron containing TRPV1 to high temperature leads to a sensation of heat.

(b) Capsaicin, one of the active ingredients in "hot" peppers, is an agonist of TRPV1. Capsaicin shows 50% activation of the TRPV1 response at a concentration of 32 nM—a property known as EC_{50}. Explain why even a very few drops of hot pepper sauce can taste very "hot" without actually burning you.

(c) Menthol, one of the active ingredients in mint, is an agonist of TRPM8 (EC_{50} = 30 *n*M) and TRPV3 (EC_{50} = 20 μM). What sensation would you expect from contact with low levels of menthol? With high levels?

19. Oncogenes, Tumor Suppressor Genes, and Tumors For each of the following situations, provide a plausible explanation for how it could lead to unrestricted cell division.

(a) Colon cancer cells often contain mutations in the gene encoding the prostaglandin E_2 receptor. PGE_2 is a growth factor required for the division of cells in the gastrointestinal tract.

(b) Kaposi sarcoma, a common tumor in people with untreated AIDS, is caused by a virus carrying a gene for a protein similar to the chemokine receptors CXCR1 and CXCR2. Chemokines are cell-specific growth factors.

(c) Adenovirus, a tumor virus, carries a gene for the protein E1A, which binds to the retinoblastoma protein, pRb. (Hint: See Fig. 12-37.)

(d) An important feature of many oncogenes and tumor suppressor genes is their cell-type specificity. For example, mutations in the PGE_2 receptor are not typically found in lung tumors. Explain this observation. (Note that PGE_2 acts through a GPCR in the plasma membrane.)

20. Mutations in Tumor Suppressor Genes and Oncogenes Explain why mutations in tumor suppressor genes are recessive (both copies of the gene must be defective for the regulation of cell division to be defective), whereas mutations in oncogenes are dominant.

21. Retinoblastoma in Children Explain why some children with retinoblastoma develop multiple tumors of the retina in both eyes, whereas

others have a single tumor in only one eye.

22. Specificity of a Signal for a Single Cell Type Discuss the validity of the following proposition. A signaling molecule (hormone, growth factor, or neurotransmitter) elicits identical responses in different types of target cells if they contain identical receptors.

Data Analysis Problem

23. Exploring Taste Sensation in Mice Pleasing tastes are an evolutionary adaptation to encourage animals to consume nutritious foods. Zhao and coauthors (2003) examined the two major pleasurable taste sensations: sweet and umami. Umami is a "distinct savory taste" triggered by amino acids, especially aspartate and glutamate, and probably encourages animals to consume protein-rich foods. Monosodium glutamate (MSG) is a flavor enhancer that exploits this sensitivity.

At the time the article was published, specific taste receptor proteins for sweet and umami had been tentatively characterized. Three such proteins were known—T1R1, T1R2, and T1R3—which function as heterodimeric receptor complexes: T1R1-T1R3 was tentatively identified as the umami receptor, and T1R2-T1R3 as the sweet receptor. It was not clear how taste sensation was encoded and sent to the brain, and two possible models had been suggested. In the cell-based model, individual taste-sensing cells express only one kind of receptor; that is, there are "sweet cells," "bitter cells," "umami cells," and so on, and each type of cell sends its information to the brain via a different nerve. The brain "knows" which taste is detected by the identity of the nerve fiber that transmits the message. In the receptor-based model, individual taste-sensing cells have several kinds of receptors and send different messages along the same nerve fiber to the brain, the message depending on which receptor is activated. Also unclear at the time was whether there was any interaction between the different taste sensations, or whether parts of one taste-sensing system were required for other taste sensations.

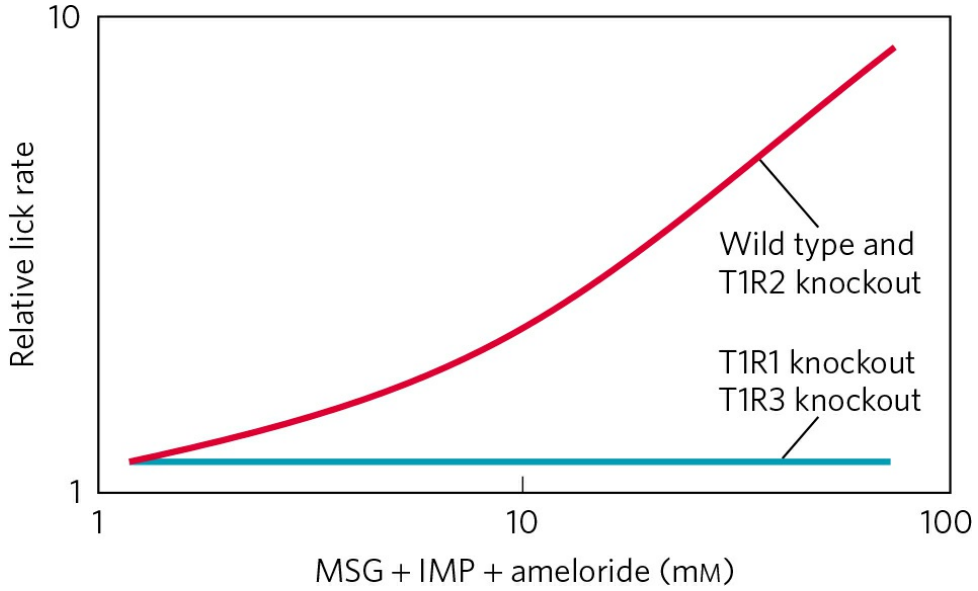
(a) Previous work had shown that different taste receptor proteins are expressed in non-overlapping sets of taste receptor cells. Which model does this support? Explain your reasoning.

Zhao and colleagues constructed a set of "knockout mice"—mice homozygous for loss-of-function alleles for one of the three receptor proteins, T1R1, T1R2, or T1R3—and double-knockout mice with nonfunctioning T1R2 and T1R3. The researchers measured the taste perception of these mice by measuring their "lick rate" of solutions containing different taste molecules. Mice will lick the spout of a feeding bottle with a pleasant-tasting solution more often than one with an unpleasant-tasting solution. The researchers measured relative lick rates: how often the mice licked a sample solution compared with water. A relative lick rate of 1 indicated no preference; <1, an aversion; and >1, a preference.

(b) All four types of knockout strains had the same responses to salt and bitter tastes as did wild-type mice. Which of the above issues did this experiment

address? What do you conclude from these results?

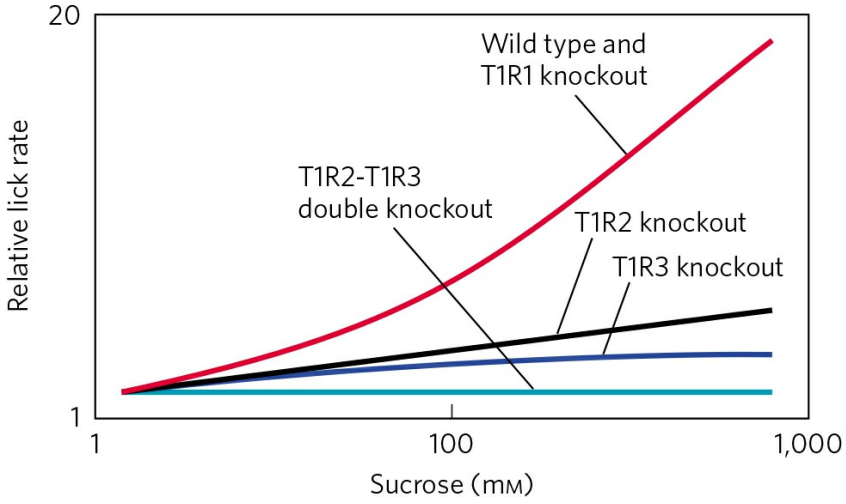
The researchers then studied umami taste reception by measuring the relative lick rates of the different mouse strains with different quantities of MSG in the feeding solution. Note that the solutions also contained inosine monophosphate (IMP), a strong potentiator of umami taste reception (and a common ingredient in ramen soups, along with MSG), and ameloride, which suppresses the pleasant salty taste imparted by the sodium of MSG. The results are shown in the graph.



(c) Are these data consistent with the umami taste receptor consisting of a heterodimer of T1R1 and T1R3? Why or why not?

(d) Which model(s) of taste encoding does this result support? Explain your reasoning.

Zhao and coworkers then performed a series of similar experiments using sucrose as a sweet taste. These results are shown below.



(e) Are these data consistent with the sweet taste receptor consisting of a heterodimer of T1R2 and T1R3? Why or why not?

(f) There were some unexpected responses at very high sucrose concentrations. How do these complicate the idea of a heterodimeric system as presented above?

In addition to sugars, humans also taste other compounds (e.g., saccharin and the peptides monellin and aspartame) as sweet; mice do not taste these as sweet. Zhao and coworkers inserted into TIR2-knockout mice a copy of the human T1R2 gene under the control of the mouse T1R2 promoter. These modified mice now tasted monellin and saccharin as sweet. The researchers then went further, adding to T1R1-knockout mice the RASSL protein—a G protein–linked receptor for the synthetic opiate spiradoline; the RASSL gene was under the control of a promoter that could be induced by feeding the mice tetracycline. These mice did not prefer spiradoline in the absence of tetracycline; in the presence of tetracycline, they showed a strong preference for nanomolar concentrations of spiradoline.

(g) Do these results strengthen your conclusions about the mechanism of taste sensation?

Reference

Zhao, G.Q., Y. Zhang, M.A. Hoon, J. Chandrashekar, I. Erlenbach, N.J.P. Ryba, and C. Zuker. 2003. The receptors for mammalian sweet and umami taste. *Cell* 115:255–266.

Further Reading is available at www.macmillanlearning.com/LehningerBiochemistry7e.