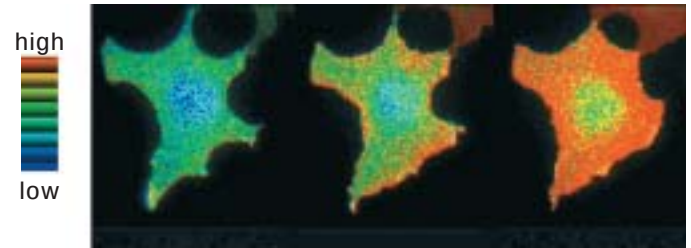


14

SIGNALING PATHWAYS THAT CONTROL GENE ACTIVITY



Fluorescence resonance energy transfer (FRET) detects time and location of activation of Ras protein in live cells triggered by epidermal growth factor. [Michiyuki Matsuda, Research Institute for Microbial Diseases, Osaka University.]

The development of all organisms requires execution of a complex program whereby specific genes are activated and repressed in specific sets of cells and in a precise time sequence. Many developmental changes in gene expression are generated by extracellular **signaling molecules** that act on cell-surface **receptors**. Most of these signals are soluble, secreted factors that act in a paracrine fashion on receiving (target) cells near the releasing cell. However, some signaling proteins are themselves attached to the cell surface, where they interact with cell-surface receptors on adjacent cells to alter the receiving cell's pattern of gene expression.

Even mature cells that are part of a differentiated tissue constantly change their patterns of gene expression. In large measure this occurs because of many different cell-surface receptors that continually receive information from extracellular signals and transduce this information into activation of specific transcription factors that stimulate or repress expression of specific target genes. Many such signaling pathways lead to alterations in the cell's metabolic activities. Liver, for example, responds to fluctuations in the levels of many hormones (e.g., insulin, glucagon, and epinephrine) by altering expression of many genes encoding enzymes of glucose and fat metabolism. Other signaling pathways influence the levels of proteins that affect the ability of cells to progress through the cell cycle and divide.

A typical mammalian cell often expresses cell-surface receptors for more than 100 different types of extracellular signaling molecules that function primarily to regulate the activity of **transcription factors** (see Figure 13-1). The signal-induced activation of transcription factors occurs by several mechanisms. In the last chapter, for instance, we saw that

stimulation of some G protein-coupled receptors leads to a rise in cAMP and the cAMP-dependent activation of protein kinase A. After translocating to the nucleus, protein kinase A phosphorylates and thereby activates the CREB transcription factor.

In this chapter, we focus on five other classes of cell-surface receptors that illustrate additional signal-induced mechanisms of activating transcription factors. Stimulation of *transforming growth factor β (TGF β) receptors* and *cytokine receptors* leads directly to activation of cytosolic transcription factors as the result of phosphorylation by a kinase that is part of the receptor or associated with it. The activated transcription factors then translocate into the nucleus and act on specific target genes. In the case of *receptor*

OUTLINE

- 14.1 TGF β Receptors and the Direct Activation of Smads
- 14.2 Cytokine Receptors and the JAK-STAT Pathway
- 14.3 Receptor Tyrosine Kinases and Activation of Ras
- 14.4 MAP Kinase Pathways
- 14.5 Phosphoinositides as Signal Transducers
- 14.6 Pathways That Involve Signal-Induced Protein Cleavage
- 14.7 Down-Modulation of Receptor Signaling

TABLE 14-1 Overview of Major Receptor Classes and Signaling Pathways

Receptor Class/Pathway*	Distinguishing Characteristics
RECEPTORS LINKED TO TRIMERIC G PROTEINS	
G protein–coupled receptors (13)	<i>Ligands:</i> Epinephrine, glucagon, serotonin, vasopressin, ACTH, adenosine, and many others (mammals); odorant molecules, light; mating factors (yeast) <i>Receptors:</i> Seven transmembrane α helices; cytosolic domain associated with a membrane-tethered trimeric G protein <i>Signal transduction:</i> (1) Second-messenger pathways involving cAMP or IP_3/DAG; (2) linked ion channels; (3) MAP kinase pathway
RECEPTORS WITH INTRINSIC OR ASSOCIATED ENZYMATIC ACTIVITY	
TGF β receptors (14, 15)	<i>Ligands:</i> Transforming growth factor β superfamily (TGFβ, BMPs), activin, inhibins (mammals); Dpp (<i>Drosophila</i>) <i>Receptors:</i> Intrinsic protein serine/threonine kinase activity in cytosolic domain (type I and II) <i>Signal transduction:</i> Direct activation of cytosolic Smad transcription factors
Cytokine receptors (14, 15)	<i>Ligands:</i> Interferons, erythropoietin, growth hormone, some interleukins (IL-2, IL-4), other cytokines <i>Receptors:</i> Single transmembrane α helix; conserved multi-β strand fold in extracellular domain; JAK kinase associated with intracellular domain <i>Signal transduction:</i> (1) Direct activation of cytosolic STAT transcription factors; (2) PI-3 kinase pathway; (3) IP_3/DAG pathway; (4) Ras-MAP kinase pathway
Receptor tyrosine kinases (14)	<i>Ligands:</i> Insulin, epidermal growth factor (EGF), fibroblast growth factor (FGF), neurotrophins, other growth factors <i>Receptor:</i> Single transmembrane α helix; intrinsic protein tyrosine kinase activity in cytosolic domain <i>Signal transduction:</i> (1) Ras–MAP kinase pathway; (2) IP_3/DAG pathway; (3) PI-3 kinase pathway
Receptor guanylyl cyclases (13)	<i>Ligands:</i> Atrial natriuretic factor and related peptide hormones <i>Receptor:</i> Single transmembrane α helix; intrinsic guanylate cyclase activity in cytosolic domain <i>Signal transduction:</i> Generation of cGMP
Receptor phosphotyrosine phosphatases	<i>Ligands:</i> Pleiotrophins, other protein hormones <i>Receptors:</i> Intrinsic phosphotyrosine phosphatase activity in cytosolic domain inhibited by ligand binding <i>Signal transduction:</i> Hydrolysis of activating phosphotyrosine residue on cytosolic protein tyrosine kinases
T-cell receptors	<i>Ligands:</i> Small peptides associated with major histocompatibility (MHC) proteins in the plasma membrane of macrophages and other antigen-presenting cells <i>Receptors:</i> Single transmembrane α helix; several protein kinases associated with cytosolic domain; found only on T lymphocytes <i>Signal transduction:</i> (1) Activation of cytosolic protein tyrosine kinases; (2) PI-3 kinase pathway; (3) IP_3/DAG pathway; (4) Ras–MAP kinase pathway

TABLE 14-1 Overview of Major Receptor Classes and Signaling Pathways

Receptor Class/Pathway*	Distinguishing Characteristics
RECEPTORS THAT ARE ION CHANNELS	
Ligand-gated ion channels (7, 13)	<i>Ligands:</i> Neurotransmitters (e.g., acetylcholine, glutamate), cGMP, physical stimuli (e.g., touch, stretching), IP₃ (receptor in ER membrane) <i>Receptors:</i> Four or five subunits with a homologous segment in each subunit lining the ion channel <i>Signal transduction:</i> (1) Localized change in membrane potential due to ion influx, (2) elevation of cytosolic Ca²⁺
PATHWAYS INVOLVING PROTEOLYSIS	
Wnt pathway (15)	<i>Ligands:</i> Secreted Wnt (mammals); Wg (<i>Drosophila</i>) <i>Receptors:</i> Frizzled (Fz) with seven transmembrane α helices; associated membrane-bound LDL receptor-related protein (Lrp) required for receptor activity <i>Signal transduction:</i> Assembly of multiprotein complex at membrane that inhibits the proteasome-mediated proteolysis of cytosolic β-catenin transcription factor, resulting in its accumulation
Hedgehog (Hh) pathway (15)	<i>Ligands:</i> Cell-tethered Hedgehog <i>Receptors:</i> Binding of Hh to Patched (Ptc), which has 12 transmembrane α helices; activation of signaling from Smoothened (Smo), with 7 transmembrane α helices <i>Signal transduction:</i> Proteolytic release of a transcriptional activator from multiprotein complex in the cytosol
Notch/Delta pathway (14, 15)	<i>Ligands:</i> Membrane-bound Delta or Serrate protein <i>Receptors:</i> Extracellular subunit of Notch receptor noncovalently associated with transmembrane-cytosolic subunit <i>Signal transduction:</i> Intramembrane proteolytic cleavage of receptor transmembrane domain with release of cytosolic segment that functions as co-activator for nuclear transcription factors
NF- κ B pathways (14, 15)	<i>Ligands:</i> Tumor necrosis factor α (TNF-α), interleukin 1 (mammals); Spätzle (<i>Drosophila</i>) <i>Receptors:</i> Various in mammals; Toll and Toll-like receptors in <i>Drosophila</i> <i>Signal transduction:</i> Phosphorylation-dependent degradation of inhibitor protein with release of active NF-κB transcription factor (Dorsal in <i>Drosophila</i>) in the cytosol
INTRACELLULAR RECEPTORS PATHWAYS	
Nitric oxide pathway (13)	<i>Ligands:</i> Nitric oxide (NO) <i>Receptor:</i> Cytosolic guanylyl cyclase <i>Signal transduction:</i> Generation of cGMP
Nuclear receptor pathways (11)	<i>Ligands:</i> Lipophilic molecules including steroid hormones, thyroxine, retinoids, and fatty acids in mammals and ecdysone in <i>Drosophila</i> <i>Receptors:</i> Highly conserved DNA-binding domain, somewhat conserved hormone-binding domain, and a variable domain; located within nucleus or cytosol <i>Signal transduction:</i> Activation of receptor's transcription factor activity by ligand binding

*Unless indicated otherwise, receptors are located in the plasma membrane. Numbers in parentheses indicate chapters in which a receptor/pathway is discussed in depth.

SOURCES: J. Gerhart, 1999, *Teratology* **60**:226, and A. Brivanlou and J. E. Darnell, 2002, *Science* **295**:813.

tyrosine kinases, binding of a **ligand** to its receptor sets into motion a cascade of intracellular events leading to activation of a cytosolic kinase that moves into the nucleus and activates one or more transcription factors by phosphorylation. Signaling from *tumor necrosis factor α* (*TNF- α*) receptors generates an active NF- κ B transcription factor by proteolytic cleavage of a cytosolic inhibitor protein, and proteolytic cleavage of a *Notch receptor* releases the receptor's cytosolic domain that then functions as a co-activator for transcription factors in the nucleus. Proteolysis also plays a role in the signaling pathways triggered by binding of protein ligands called Wnt and Hedgehog (Hh) to their receptors. We cover these two pathways, which play a major role during **development** and **differentiation**, in Chapter 15.

For simplicity, we often describe the various receptor classes independently, concentrating on the major pathway of **signal transduction** initiated by each class of receptor. However, as shown in Table 14-1, several classes of receptors can transduce signals by more than one pathway. Moreover, many genes are regulated by multiple transcription factors, each of which can be activated by one or more extracellular signals. Especially during early development, such cross talk between signaling pathways and the resultant sequential alterations in the pattern of gene expression eventually can become so extensive that the cell assumes a different developmental fate.

Researchers have employed a variety of experimental approaches and systems to identify and study the function of extracellular signaling molecules, receptors, and intracellular signal-transduction proteins. For instance, the secreted signaling protein Hedgehog (Hh) and its receptor were first identified in *Drosophila* mutants with developmental defects. Subsequently the human and mouse homologs of these proteins were cloned and shown to participate in a number of important signaling events during differentiation. Some signal-transduction proteins were first identified when gain-of-function mutations in the genes encoding them or overexpression of the normal protein caused abnormal cell proliferation leading to malignancy. A mutant Ras protein exhibiting unregulated (i.e., **constitutive**) activity was identified in this way; wild-type Ras later was found to be a key player in many signaling pathways. Numerous extracellular signaling molecules initially were purified from cell extracts based on their ability to stimulate growth and proliferation of specific cell types. These few examples illustrate the importance of studying signaling pathways both genetically—in flies, mice, worms, yeasts, and other organisms—and biochemically.

14.1 TGF β Receptors and the Direct Activation of Smads

A number of related extracellular signaling molecules that play widespread roles in regulating development in both invertebrates and vertebrates constitute the *transforming*

growth factor β (TGF β) superfamily. One member of this superfamily, *bone morphogenetic protein* (BMP), initially was identified by its ability to induce bone formation in cultured cells. Now called BMP7, it is used clinically to strengthen bone after severe fractures. Of the numerous BMP proteins subsequently recognized, many help induce key steps in development, including formation of mesoderm and the earliest blood-forming cells.

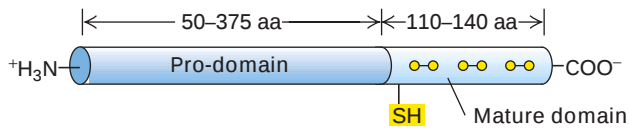
Another member of the TGF β superfamily, now called TGF β -1, was identified on the basis of its ability to induce a transformed phenotype of certain cells in culture. However, the three human TGF β isoforms that are known all have potent antiproliferative effects on many types of mammalian cells. Loss of TGF β receptors or certain intracellular signal-transduction proteins in the TGF β pathway, thereby releasing cells from this growth inhibition, frequently occurs in human tumors. TGF β proteins also promote expression of cell-adhesion molecules and extracellular-matrix molecules. TGF β signals certain types of cells to synthesize and secrete growth factors that can, on balance, overcome the normal TGF β -induced growth inhibition; this explains why TGF β was originally detected as a growth factor. A *Drosophila* homolog of TGF β , called *Dpp protein*, controls dorsoventral patterning in fly embryos, as we detail in Chapter 15. Other mammalian members of the TGF β superfamily, the activins and inhibins, affect early development of the genital tract.

Despite the complexity of cellular effects induced by various members of the TGF β superfamily, the signaling pathway is basically a simple one. Once activated, receptors for these ligands directly phosphorylate and activate a particular type of transcription factor. The response of a given cell to this activated transcription factor depends on the constellation of other transcription factors it already contains.

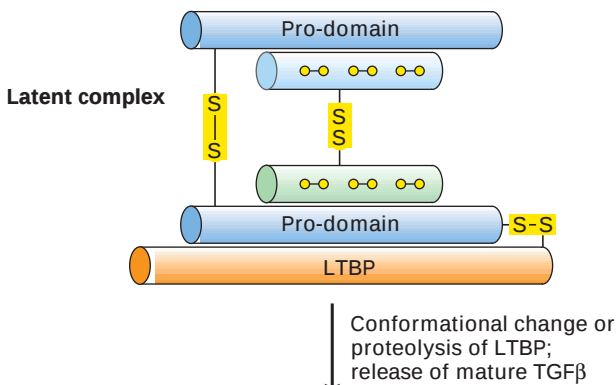
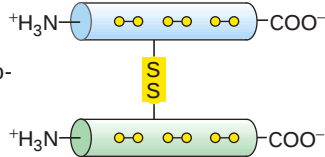
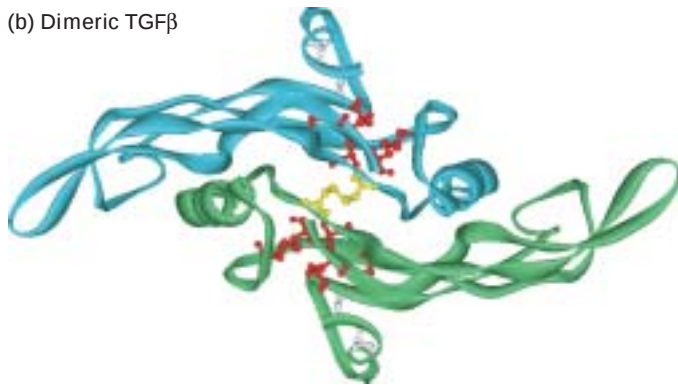
TGF β Is Formed by Cleavage of a Secreted Inactive Precursor

In humans TGF β consists of three protein isoforms, TGF β -1, TGF β -2, and TGF β -3, each encoded by a unique gene and expressed in both a tissue-specific and developmentally regulated fashion. Each TGF β isoform is synthesized as part of a larger precursor that contains a pro-domain. This domain is cleaved from but remains noncovalently associated with the mature domain after the protein is secreted. Most secreted TGF β is stored in the extracellular matrix as a latent, inactive complex containing the cleaved TGF β precursor and a covalently bound TGF β -binding protein called *Latent TGF β Binding Protein*, or *LTBP*. Binding of LTBP by the matrix protein thrombospondin or by certain cell-surface **integrins** triggers a conformational change in LTBP that causes release of the mature, active dimeric TGF β . Alternatively, digestion of the binding proteins by matrix metalloproteases can result in activation of TGF β (Figure 14-1a).

The monomeric form of TGF β growth factors contains 110–140 amino acids and has a compact structure with four antiparallel β strands and three conserved intramolecular

(a) Formation of mature, dimeric TGF β Secreted TGF β precursor

Proteolytic cleavage
Binding by LTBP

Mature form
(homo- or hetero-
dimer)(b) Dimeric TGF β 

disulfide linkages (Figure 14-1b). These form a structure, called a *cystine knot*, that is relatively resistant to denaturation. An additional N-terminal cysteine in each monomer links TGF β monomers into functional homodimers and heterodimers. Much of the sequence variation among different TGF β proteins is observed in the N-terminal regions, the loops joining the β strands, and the α helices. Different heterodimeric combinations may increase the functional diversity of these proteins beyond that generated by differences in the primary sequence of the monomer.

FIGURE 14-1 Formation and structure of TGF β superfamily of signaling molecules.

(a) TGF β precursors are cleaved soon after being secreted. The pro-domain and mature domain are stored in the extracellular matrix in a complex that also contains latent TGF β -binding protein (LTBP). The mature domain contains six conserved cysteine residues (yellow circles), which form three intrachain disulfide bonds and also a single disulfide bond connecting two monomers. Following proteolysis or a conformational change in LTBP, the active homo- or heterodimeric protein is released. (b) In this ribbon diagram of mature TGF β dimer, the two subunits are shown in green and blue. Disulfide-linked cysteine residues are shown in ball-and-stick form. The three intrachain disulfide linkages (red) in each monomer form a cystine-knot domain, which is resistant to degradation. [Part (a) see J. Massagué and Y.-G. Chen, 2000, *Genes and Devel.* **14**:627; part (b) from S. Daopin et al., 1992, *Science* **257**:369.]

TGF β Signaling Receptors Have Serine/Threonine Kinase Activity

To identify the cell-surface TGF β receptors, investigators first reacted the purified growth factor with the radioisotope iodine-125 (^{125}I) under conditions such that the radioisotope covalently binds to exposed tyrosine residues. The ^{125}I -labeled TGF β protein was incubated with cultured cells, and the incubation mixture then was treated with a chemical agent that covalently cross-linked the labeled TGF β to its receptors on the cell surface. Purification of the labeled receptors revealed three different polypeptides with apparent molecular weights of 55, 85, and 280 kDa, referred to as types *RI*, *RII*, and *RIII* TGF β receptors, respectively.

The most abundant TGF β receptor, *RIII*, is a cell-surface **proteoglycan**, also called β -*glycan*, which binds and concentrates TGF β near the cell surface. The type I and type II receptors are dimeric transmembrane proteins with serine/threonine kinases as part of their cytosolic domains. *RII* is a constitutively active kinase that phosphorylates itself in the absence of TGF β . Binding of TGF β induces the formation of complexes containing two copies each of *RI* and *RII*. An *RII* subunit then phosphorylates serine and threonine residues in a highly conserved sequence of the *RI* subunit adjacent to the cytosolic face of the plasma membrane, thereby activating the *RI* kinase activity.

Activated Type I TGF β Receptors Phosphorylate Smad Transcription Factors

Researchers identified the transcription factors downstream from TGF β receptors in *Drosophila* from genetic studies similar to those used to dissect receptor tyrosine kinase pathways (see Section 14.3). These transcription factors in *Drosophila* and the related vertebrate proteins are now called **Smads**. Three types of Smad proteins function in the TGF β signaling pathway: receptor-regulated Smads (R-Smads), co-Smads, and inhibitory or antagonistic Smads (I-Smads).

As depicted in Figure 14-2, R-Smads contain two domains, MH1 and MH2, separated by a flexible linker region. The N-terminal MH1 domain contains the specific DNA-binding segment and also a sequence called the *nuclear-localization signal* (NLS) that is required for protein transport into the nucleus (Chapter 12). When R-Smads are in their inactive, nonphosphorylated state, the NLS is masked and the MH1 and MH2 domains associate in such a way that they cannot bind to DNA or to a co-Smad. Phosphorylation of three serine residues near the C-terminus of an R-Smad (Smad2 or Smad3) by activated type I TGF β re-

ceptors separates the domains, permitting binding of importin β to the NLS. Simultaneously a complex containing two molecules of Smad3 (or Smad2) and one molecule of a co-Smad (Smad4) forms in the cytosol. This complex is stabilized by binding of two phosphorylated serines in each Smad3 to phosphoserine-binding sites in both the Smad3 and the Smad4 MH2 domains. The bound importin β then mediates translocation of the heteromeric R-Smad/co-Smad complexes into the nucleus. After importin β dissociates inside the nucleus, the Smad2/Smad4 or Smad3/Smad4 complexes cooperate with other transcription factors to activate transcription of specific target genes.

Within the nucleus R-Smads are continuously being dephosphorylated, which results in the dissociation of the R-Smad/co-Smad complex and export of these Smads from the nucleus. Because of this continuous nucleocytoplasmic shuttling of the Smads, the concentration of active Smads within the nucleus closely reflects the levels of activated TGF β receptors on the cell surface.

Virtually all mammalian cells secrete at least one TGF β isoform, and most have TGF β receptors on their surface. However, because different types of cells contain different sets of transcription factors with which the activated Smads can bind, the cellular responses induced by TGF β vary among cell types. In epithelial cells and fibroblasts, for example, TGF β induces expression not only of extracellular-

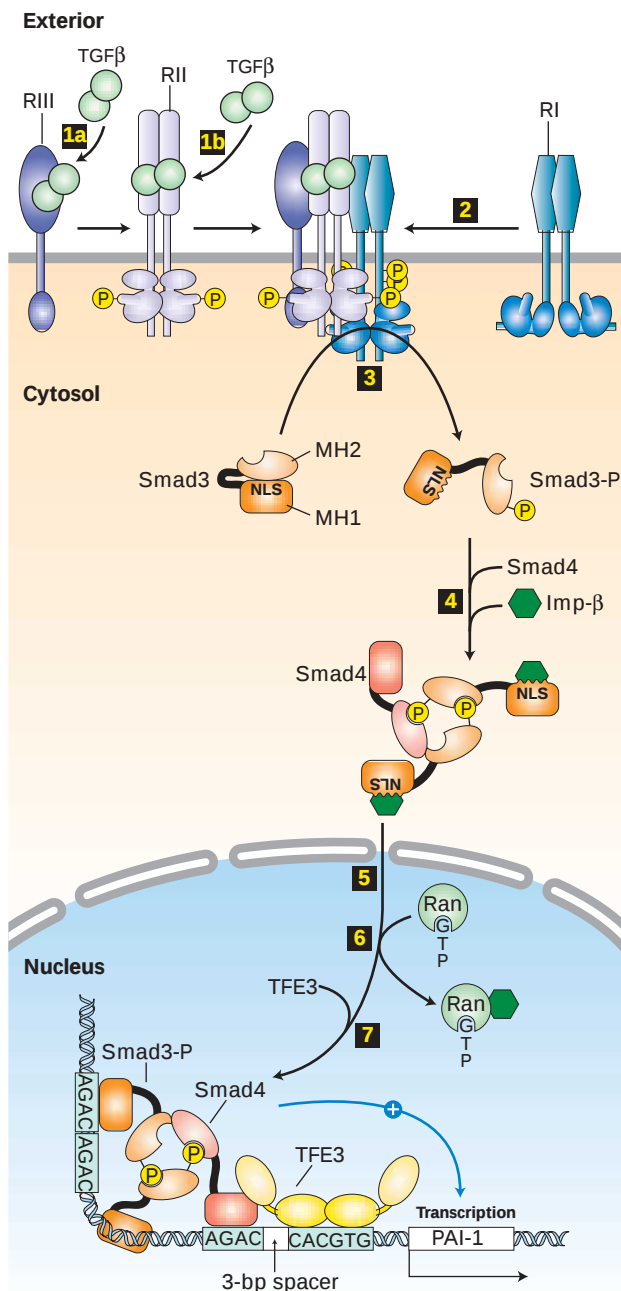


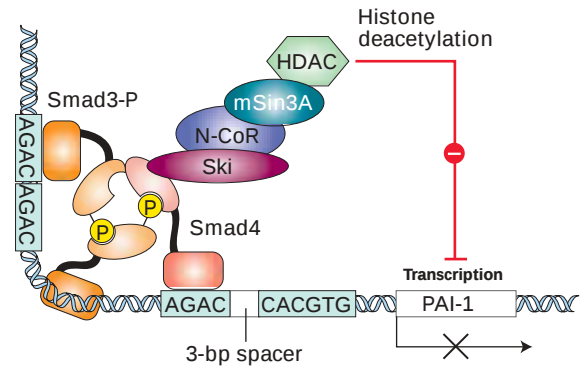
FIGURE 14-2 TGF β -Smad signaling pathway. Step **1a**: In some cells, TGF β binds to the type III TGF β receptor (RIII), which presents it to the type II receptor (RII). Step **1b**: In other cells, TGF β binds directly to RII, a constitutively phosphorylated and active kinase. Step **2**: Ligand-bound RII recruits and phosphorylates the juxtamembrane segment of the type I receptor (RI), which does not directly bind TGF β . This releases the inhibition of RI kinase activity that otherwise is imposed by the segment of RI between the membrane and kinase domain. Step **3**: Activated RI then phosphorylates Smad3 (shown here) or another R-Smad, causing a conformational change that unmasks its nuclear-localization signal (NLS). Step **4**: Two phosphorylated molecules of Smad3 interact with a co-Smad (Smad4), which is not phosphorylated, and with importin β (Imp- β), forming a large cytosolic complex. Steps **5** and **6**: After the entire complex translocates into the nucleus, Ran-GTP causes dissociation of Imp- β as discussed in Chapter 12. Step **7**: A nuclear transcription factor (e.g., TFE3) then associates with the Smad3/Smad4 complex, forming an activation complex that cooperatively binds in a precise geometry to regulatory sequences of a target gene. Shown at the bottom is the activation complex for the gene encoding plasminogen activator inhibitor (PAI-1). See the text for additional details. [See Z. Xiao et al., 2000, *J. Biol. Chem.* **275**:23425; J. Massagué and D. Wotton, 2000, *EMBO J.* **19**:1745; X. Hua et al., 1999, *Proc. Nat'l. Acad. Sci. USA* **96**:13130; and A. Moustakas and C.-H. Heldin, 2002, *Genes Devel.* **16**:1867.]

matrix proteins (e.g., collagens) but also of proteins that inhibit serum proteases, which otherwise would degrade the matrix. The latter category includes plasminogen activator inhibitor 1 (PAI-1). Transcription of the PAI-1 gene requires formation of a complex of the transcription factor TFE3 with the Smad3/Smad4 complex and binding of all these proteins to specific sequences within the regulatory region of the PAI-1 gene (see Figure 14-2, *bottom*). By partnering with other transcription factors, Smad2/Smad4 and Smad3/Smad4 complexes induce expression of proteins such as p15, which arrests the cell cycle at the G₁ stage and thus blocks cell proliferation (Chapter 21). These Smad complexes also repress transcription of the *myc* gene, thereby reducing expression of many growth-promoting genes whose transcription normally is activated by Myc.

The various growth factors in the TGF β superfamily bind to their own receptors and activate different sets of Smad proteins, resulting in different cellular responses. The specificity exhibited by these related receptors is a common phenomenon in intercellular signaling, and the TGF β signaling pathway provides an excellent example of one strategy for achieving such response specificity. As just discussed, for instance, binding of any one TGF β isoform to its specific receptors leads to phosphorylation of Smad2 or Smad3, formation of Smad2/Smad4 or Smad3/Smad4 complexes, and eventually transcriptional activation of specific target genes (e.g., the PAI-1 gene). On the other hand, BMP proteins, which also belong to the TGF β superfamily, bind to and activate a different set of receptors, leading to phosphorylation of Smad1, its dimerization with Smad4, and activation of specific transcriptional responses by Smad1/Smad4. These responses are distinct from those induced by Smad2/Smad4 or Smad3/Smad4.

Oncoproteins and I-Smads Regulate Smad Signaling via Negative Feedback Loops

Smad signaling is regulated by additional intracellular proteins, including two cytosolic proteins called *SnoN* and *Ski* (*Ski* stands for “Sloan-Kettering Cancer Institute”). These proteins were originally identified as **oncoproteins** because they cause abnormal cell proliferation when overexpressed in cultured fibroblasts. How they accomplish this was not understood until years later when *SnoN* and *Ski* were found to bind to the Smad2/Smad4 or Smad3/Smad4 complexes formed after TGF β stimulation. *SnoN* and *Ski* do not affect the ability of the Smad complexes to bind to DNA control regions. Rather, they block transcription activation by the bound Smad complexes, thereby rendering cells resistant to the growth-inhibitory actions normally induced by TGF β (Figure 14-3). Interestingly, stimulation by TGF β causes the rapid degradation of *Ski* and *SnoN*, but after a few hours, expression of both *Ski* and *SnoN* becomes strongly induced. The increased levels of these proteins are thought to dampen long-term signaling effects due to continued exposure to TGF β .



▲ **FIGURE 14-3 Schematic model of Ski-mediated down-regulation of the response to TGF β stimulation.** Ski binds to Smad4 in Smad3/Smad4 or Smad2/Smad4 (not shown) signaling complexes and may partially disrupt interactions between the Smad proteins. Ski also recruits a protein termed *N-CoR* that binds directly to *mSin3A*, which in turn interacts with histone deacetylase (*HDAC*), an enzyme that promotes histone deacetylation (Chapter 11). As a result, transcription activation induced by TGF β and mediated by Smad complexes is shut down. [See S. Stroschein et al., 1999, *Science* **286**:771; X. Liu et al., 2001, *Cytokine and Growth Factor Rev.* **12**:1; and J.-W. Wu et al., 2002, *Cell* **111**:357.]

Among the proteins induced after TGF β stimulation are the I-Smads, especially Smad7. Smad7 blocks the ability of activated type I receptors to phosphorylate R-Smad proteins. In this way Smad7, like *Ski* and *SnoN*, participates in a negative feedback loop; its induction serves to inhibit intracellular signaling by long-term exposure to the stimulating hormone. In later sections we see how signaling by other cell-surface receptors is also controlled by negative feedback loops.

Loss of TGF β Signaling Contributes to Abnormal Cell Proliferation and Malignancy



Many human tumors contain inactivating mutations in either TGF β receptors or Smad proteins, and thus are resistant to growth inhibition by TGF β (see Figure 23-20). Most human pancreatic cancers, for instance, contain a deletion in the gene encoding Smad4 and thus cannot induce p15 and other cell-cycle inhibitors in response to TGF β . This mutation-defined gene originally was called *DPC* (*deleted in pancreatic cancer*). Retinoblastoma, colon and gastric cancer, hepatoma, and some T- and B-cell malignancies are also unresponsive to TGF β growth inhibition. This loss of responsiveness correlates with loss of type I or type II TGF β receptors; responsiveness to TGF β can be restored by recombinant expression of the “missing” protein. Mutations in Smad2 also commonly occur in several types of human tumors. Not only is TGF β signaling essential

for controlling cell proliferation, as these examples show, but it also causes some cells to differentiate along specific pathways, as discussed in Chapter 15. ■

KEY CONCEPTS OF SECTION 14.1

TGF β Receptors and the Direct Activation of Smads

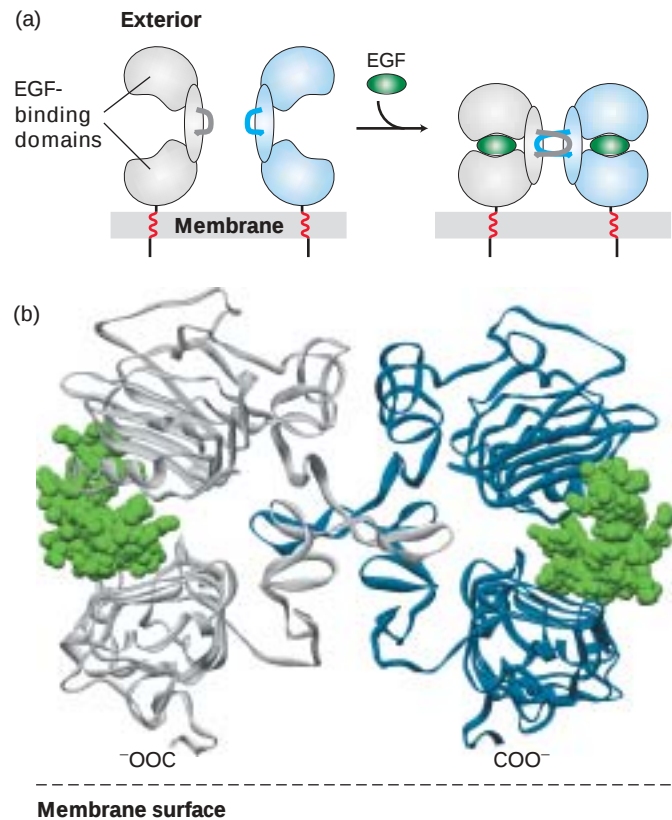
- TGF β is produced as an inactive precursor that is stored in the extracellular matrix. Several mechanisms can release the active, mature dimeric growth factor (see Figure 14-1).
- Stimulation by TGF β leads to activation of the intrinsic serine/threonine kinase activity in the cytosolic domain of the type I (RI) receptor, which then phosphorylates an R-Smad, exposing a nuclear-localization signal.
- After phosphorylated R-Smad binds a co-Smad, the resulting complex translocates into the nucleus, where it interacts with various transcription factors to induce expression of target genes (see Figure 14-2).
- Oncoproteins (e.g., Ski and SnoN) and I-Smads (e.g., Smad7) act as negative regulators of TGF β signaling.
- TGF β signaling generally inhibits cell proliferation. Loss of various components of the signaling pathway contributes to abnormal cell proliferation and malignancy.

14.2 Cytokine Receptors and the JAK-STAT Pathway

We turn now to a second important class of cell-surface receptors, the **cytokine receptors**, whose cytosolic domains are closely associated with a member of a family of cytosolic protein tyrosine kinases, the *JAK kinases*. A third class of receptors, the **receptor tyrosine kinases (RTKs)**, contain intrinsic protein tyrosine kinase activity in their cytosolic domains. The mechanisms by which cytokine receptors and receptor tyrosine kinases become activated by ligands are very similar, and there is considerable overlap in the intracellular signal-transduction pathways triggered by activation of receptors in both classes. In this section, we first describe some similarities in signaling from these two receptor classes. We then discuss the JAK-STAT pathway, which is initiated mainly by activation of cytokine receptors.

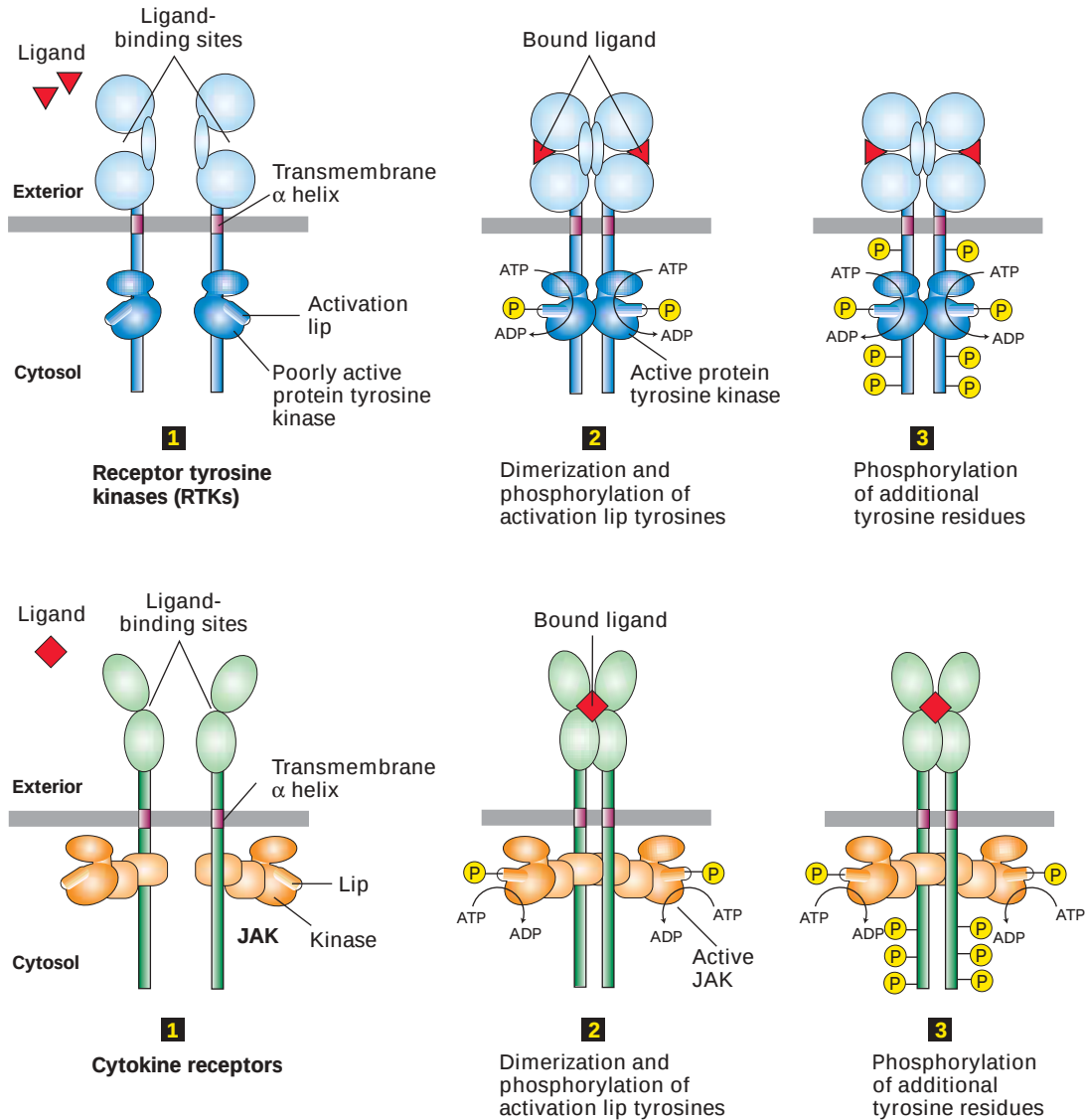
Cytokine Receptors and Receptor Tyrosine Kinases Share Many Signaling Features

Ligand binding to both cytokine receptors and RTKs triggers formation of functional dimeric receptors. In some cases, the ligand induces association of two monomeric



▲ **FIGURE 14-4 Dimerization of the receptor for epidermal growth factor (EGF), a receptor tyrosine kinase.** (a) Schematic depiction of the extracellular and transmembrane domains of the EGF receptor. Binding of one EGF molecule to a monomeric receptor causes an alteration in the structure of a loop located between the two EGF-binding domains. Dimerization of two identical ligand-bound receptor monomers in the plane of the membrane occurs primarily through interactions between the two “activated” loop segments. (b) Structure of the dimeric EGF receptor’s extracellular domain bound to transforming growth factor α (TGF α), a homolog of EGF. The EGF receptor extracellular domains are shown in white (*left*) and blue (*right*). The two smaller TGF α molecules are colored green. Note the interaction between the “activated” loop segments in the two receptors. [Part (a) adapted from J. Schlessinger, 2002, *Cell* **110**:669; part (b) from T. Garrett et al., 2002, *Cell* **110**:763.]

receptor subunits diffusing in the plane of the plasma membrane (Figure 14-4). In others, the receptor is a dimer in the absence of ligand, and ligand binding alters the conformation of the extracellular domains of the two subunits. In either case, formation of a functional dimeric receptor causes one of the poorly active cytosolic kinases to phosphorylate a particular tyrosine residue in the *activation lip* of the second kinase. This phosphorylation activates kinase activity and leads to phosphorylation of the second kinase in the dimer, as well as several tyrosine



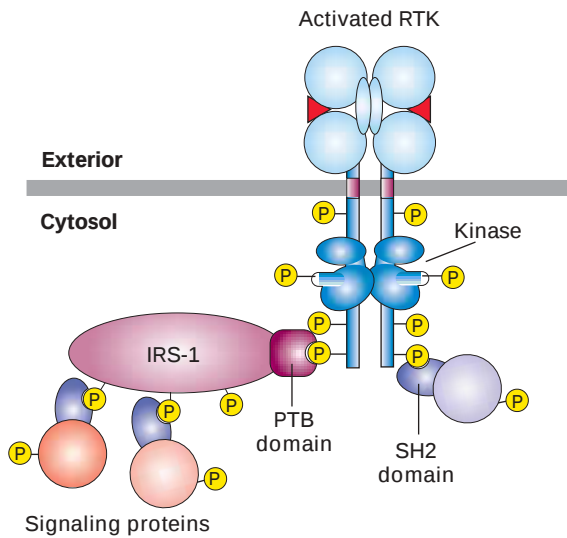
▲ **FIGURE 14-5 General structure and ligand-induced activation of receptor tyrosine kinases (RTKs) and cytokine receptors.** The cytosolic domain of RTKs contains a protein tyrosine kinase catalytic site, whereas the cytosolic domain of cytokine receptors associates with a separate JAK kinase (step 1). In both types of receptor, ligand binding causes a conformational change that promotes formation of a functional dimeric receptor, bringing together two intrinsic or associated

kinases, which then phosphorylate each other on a tyrosine residue in the activation lip (step 2). Phosphorylation causes the lip to move out of the kinase catalytic site, thus allowing ATP or a protein substrate to bind. The activated kinase then phosphorylates other tyrosine residues in the receptor's cytosolic domain (step 3). The resulting phosphotyrosines function as docking sites for various signal-transduction proteins (see Figure 14-6).

residues in the cytosolic domain of the receptor (Figure 14-5). As we see later, phosphorylation of residues in the activation loop is a general mechanism by which many kinases are activated.

Certain phosphotyrosine residues formed in activated cytokine receptors and RTKs serve as binding, or “docking,” sites for *SH2 domains* or *PTB domains*, which are present in a large array of intracellular signal-transduction proteins.

Once they are bound to an activated receptor, some signal-transduction proteins are phosphorylated by the receptor's intrinsic or associated kinase to achieve their active form. Binding of other signal-transduction proteins, present in the cytosol in unstimulated cells, positions them near their substrates localized in the plasma membrane. Both mechanisms can trigger downstream signaling. Several cytokine receptors (e.g., the IL-4 receptor) and RTKs (e.g., the insulin receptor)



▲ **FIGURE 14-6 Recruitment of signal-transduction proteins to the cell membrane by binding to phosphotyrosine residues in activated receptors.** Cytosolic proteins with SH2 (purple) or PTB (maroon) domains can bind to specific phosphotyrosine residues in activated RTKs (shown here) or cytokine receptors. In some cases, these signal-transduction proteins then are phosphorylated by the receptor's intrinsic or associated protein tyrosine kinase, enhancing their activity. Certain RTKs and cytokine receptors utilize multidocking proteins such as IRS-1 to increase the number of signaling proteins that are recruited and activated. Subsequent phosphorylation of the IRS-1 by receptor kinase activity creates additional docking sites for SH2-containing signaling proteins.

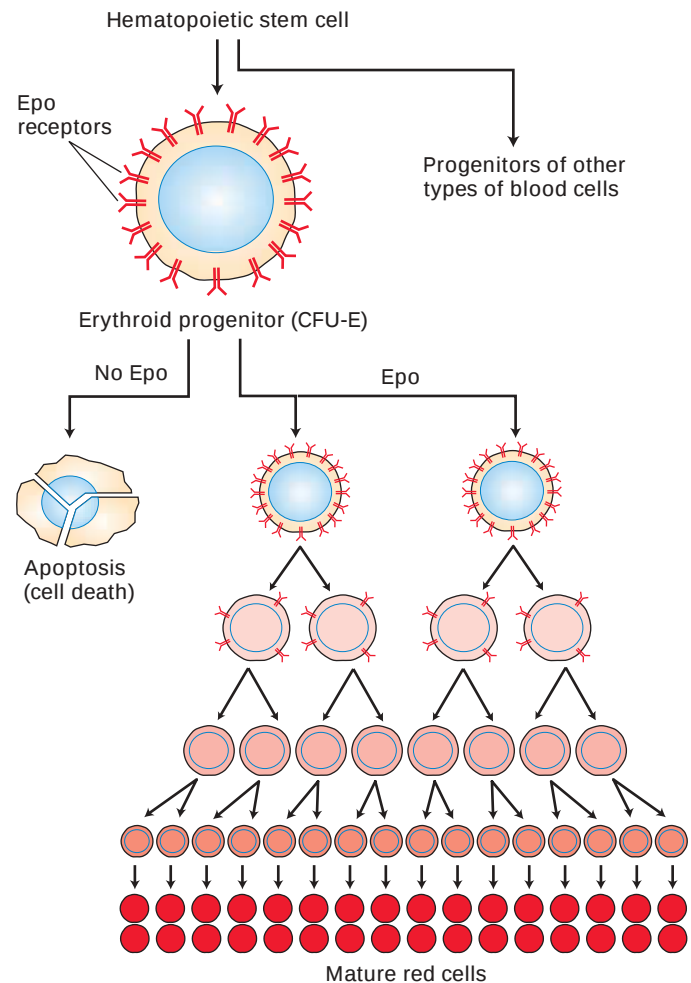
► **FIGURE 14-7 Role of erythropoietin in formation of red blood cells (erythrocytes).** Erythroid progenitor cells, called *colony-forming units erythroid* (CFU-E), are derived from hematopoietic stem cells, which also give rise to progenitors of other blood cell types. In the absence of erythropoietin (Epo), CFU-E cells undergo apoptosis. Binding of erythropoietin to its receptors on a CFU-E induces transcription of several genes whose encoded proteins prevent programmed cell death (apoptosis), allowing the cell to survive and undergo a program of three to five terminal cell divisions. Epo stimulation also induces expression of erythrocyte-specific proteins such as the globins, which form hemoglobin, and the membrane proteins glycophorin and anion-exchange protein. The Epo receptor and other membrane proteins are lost from these cells as they undergo differentiation. If CFU-E cells are cultured with erythropoietin in a semisolid medium (e.g., containing methylcellulose), daughter cells cannot move away, and thus each CFU-E produces a colony of 30–100 erythroid cells, hence its name.

[See M. Socolovsky et al., 2001, *Blood* 98:3261.]

bind IRS1 or other multidocking proteins via a PTB domain in the docking protein (Figure 14-6). The activated receptor then phosphorylates the bound docking protein, forming many phosphotyrosines that in turn serve as docking sites for SH2-containing signaling proteins. Some of these proteins in turn may also be phosphorylated by the activated receptor.

Cytokines Influence Development of Many Cell Types

The **cytokines** form a family of relatively small, secreted proteins (generally containing about 160 amino acids) that control many aspects of growth and differentiation of specific types of cells. During pregnancy prolactin, for example, induces epithelial cells lining the immature ductules of the mammary gland to differentiate into the acinar cells that produce milk proteins and secrete them into the ducts. Another cytokine, interleukin 2 (IL-2), is essential for proliferation and functioning of the T cells of the immune system; its close relative IL-4 is essential for formation of functional antibody-producing B cells. Some cytokines, such as interferon α , are produced and secreted by many types of cells fol-



lowing virus infection. The secreted interferons act on nearby cells to induce enzymes that render these cells more resistant to virus infection.

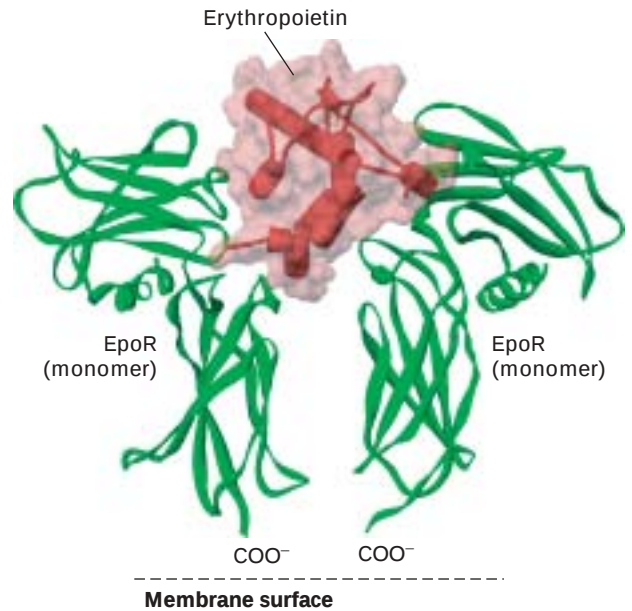


Many cytokines induce formation of important types of blood cells. For instance, granulocyte colony stimulating factor (G-CSF) induces a particular type of progenitor cell in the bone marrow to divide several times and then differentiate into granulocytes, the type of white blood cell that inactivates bacteria and other pathogens. Because many cancer therapies reduce granulocyte formation by the body, G-CSF often is administered to patients to stimulate proliferation and differentiation of granulocyte progenitor cells, thus restoring the normal level of granulocytes in the blood. Thrombopoietin, a “cousin” of G-CSF, similarly acts on megakaryocyte progenitors to divide and differentiate into megakaryocytes. These then fragment into the cell pieces called *platelets*, which are critical for blood clotting. ■

Another related cytokine, **erythropoietin (Epo)**, triggers production of red blood cells by inducing the proliferation and differentiation of erythroid progenitor cells in the bone marrow (Figure 14-7). Erythropoietin is synthesized by kidney cells that monitor the concentration of oxygen in the blood. A drop in blood oxygen signifies a lower than optimal level of erythrocytes (red blood cells), whose major function is to transport oxygen complexed to hemoglobin. By means of the oxygen-sensitive transcription factor HIF-1 α , the kidney cells respond to low oxygen by synthesizing more erythropoietin and secreting it into the blood (see Figure 15-9). As the level of erythropoietin rises, more and more erythroid progenitors are saved from death, allowing each to produce ≈ 50 or so red blood cells in a period of only two days. In this way, the body can respond to the loss of blood by accelerating the production of red blood cells.

All Cytokines and Their Receptors Have Similar Structures and Activate Similar Signaling Pathways

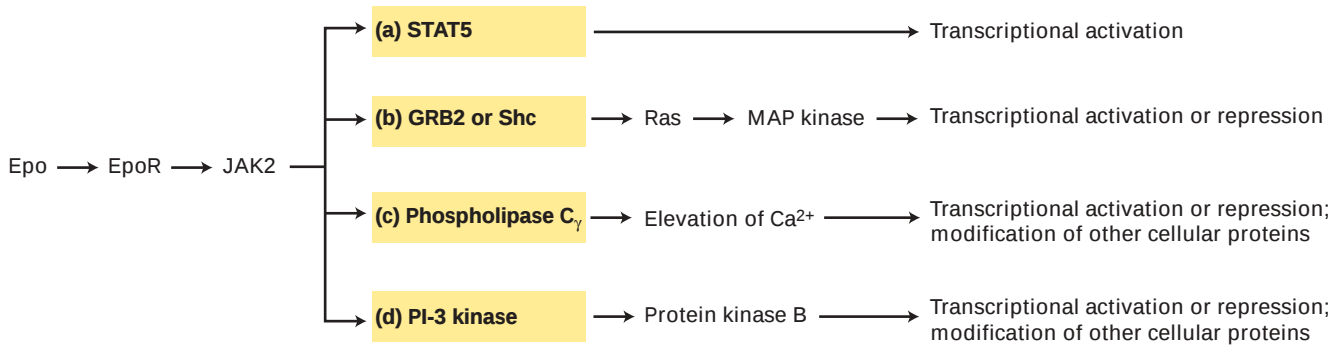
Strikingly, all cytokines have a similar tertiary structure, consisting of four long conserved α helices folded together in a specific orientation. Similarly, the structures of all cytokine receptors are quite similar, with their extracellular domains constructed of two subdomains, each of which contains seven conserved β strands folded together in a characteristic fashion. The interaction of erythropoietin with the dimeric erythropoietin receptor (EpoR), depicted in Figure 14-8, exemplifies the binding of a cytokine to its receptor. The structural homology among cytokines is evidence that they all evolved from a common ancestral protein. Likewise, the various receptors undoubtedly evolved from a single common ancestor.



▲ **FIGURE 14-8 Structure of erythropoietin bound to the extracellular domains of a dimeric erythropoietin receptor (EpoR).** Erythropoietin contains four conserved long α helices that are folded in a particular arrangement. The extracellular domain of an EpoR monomer is constructed of two subdomains, each of which contains seven conserved β strands folded in a characteristic fashion. Side chains of residues on two of the helices in erythropoietin contact loops on one EpoR monomer, while residues on the two other Epo helices bind to the same loop segments in a second receptor monomer, thereby stabilizing the dimeric receptor. The structures of other cytokines and their receptors are similar to erythropoietin and EpoR. [Adapted from R. S. Syed et al., 1998, *Nature* **395**:511]

Whether or not a cell responds to a particular cytokine depends simply on whether or not it expresses the corresponding (cognate) receptor. Although all cytokine receptors activate similar intracellular signaling pathways, the response of any particular cell to a cytokine signal depends on the cell’s constellation of transcription factors, chromatin structures, and other proteins relating to the developmental history of the cell. If receptors for prolactin or thrombopoietin, for example, are expressed experimentally in an erythroid progenitor cell, the cell will respond to these cytokines by dividing and differentiating into red blood cells, not into mammary cells or megakaryocytes.

Figure 14-9 summarizes the intracellular signaling pathways activated when the EpoR binds erythropoietin. Stimulation of other cytokine receptors by their specific ligands activates similar pathways. All these pathways eventually lead to activation of transcription factors, causing an increase or decrease in expression of particular target genes. Here we focus on the *JAK-STAT pathway*; the other pathways are discussed in later sections.



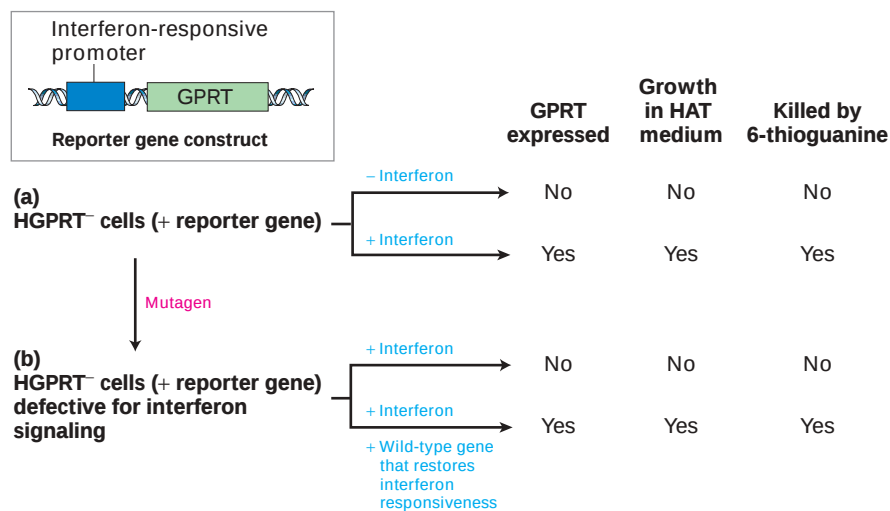
▲ **FIGURE 14-9 Overview of signal-transduction pathways triggered by ligand binding to the erythropoietin receptor (EpoR), a typical cytokine receptor.** Four major pathways can transduce a signal from the activated, phosphorylated EpoR-JAK complex (see Figure 14-5, *bottom*). Each pathway ultimately regulates transcription of different sets of genes. (a) In the most direct pathway, the transcription factor STAT5 is phosphorylated

and activated directly in the cytosol. (b) Binding of linker proteins (GRB2 or Shc) to an activated EpoR leads to activation of the Ras–MAP kinase pathway. (c, d) Two phosphoinositide pathways are triggered by recruitment of phospholipase C_γ and PI-3 kinase to the membrane following activation of EpoR. Elevated levels of Ca²⁺ and activated protein kinase B also modulate the activity of cytosolic proteins that are not involved in control of transcription.

Somatic Cell Genetics Revealed JAKs and STATs as Essential Signal-Transduction Proteins

Soon after the discovery and cloning of cytokines, most of their receptors were isolated by expression cloning or other strategies. Elucidation of the essential components of their intracellular signaling pathways, however, awaited develop-

ment of new types of genetic approaches using cultured mammalian cells. In these studies, a bacterial **reporter gene** encoding guanine phosphoribosyl transferase (GPRT) was linked to an upstream interferon-responsive promoter. The resulting construct was introduced into cultured mammalian cells that were genetically deficient in the human homolog HGPRT. GPRT or HGPRT is necessary for incorporation of purines



▲ **EXPERIMENTAL FIGURE 14-10 Mutagenized cells carrying an interferon-responsive reporter gene were used to identify JAKs and STATs as essential signal-transduction proteins.** A reporter gene was constructed consisting of an interferon-responsive promoter upstream of the bacterial gene encoding GPRT, a key enzyme in the purine salvage pathway (see Figure 6-39). (a) Introduction of this construct into mammalian cells lacking the mammalian homolog HGPRT yielded reporter cells that grew in HAT medium and were killed by 6-thioguanine in the presence but not the absence of interferon. (b) Following

treatment of reporter cells with a mutagen, cells with defects in the signaling pathway initiated by interferon do not induce GPRT in response to interferon and thus cannot incorporate the toxic purine 6-thioguanine. Restoration of interferon responsiveness by functional complementation with wild-type DNA clones identified genes encoding JAKs and STATs. See the text for details. [See R. McKendry et al., 1991, *Proc. Nat'l. Acad. Sci. USA* **88**:11455; D. Watling et al., 1993, *Nature* **366**:166; and G. Stark and A. Gudkov, 1999, *Human Mol. Genet.* **8**:1925.]

in the culture medium into ribonucleotides and then into DNA or RNA. As shown in Figure 14-10a, HGPRT-negative cells carrying the reporter gene responded to interferon treatment by expressing GPRT and thus acquiring the ability to grow in HAT medium. This medium does not allow growth of cells lacking GPRT or HGPRT, since synthesis of purines by the cells is blocked by aminopterin (the A in HAT), and thus DNA synthesis is dependent on incorporation of purines from the culture medium (see Figure 6-39). Simultaneously the cells acquired sensitivity to killing by the purine analog 6-thioguanine, which is converted into the corresponding ribonucleotide by GPRT; incorporation of this purine into DNA in place of guanosine eventually causes cell death.

The reporter cells were then heavily treated with mutagens in an attempt to inactivate both alleles of the genes encoding critical signal-transduction proteins in the interferon signaling pathway. Researchers looked for mutant cells that expressed the interferon receptor (as evidenced by the cell's ability to bind radioactive interferon) but did not express GPRT in response to interferon and thus survived killing by 6-thioguanine when cells were cultured in the presence of interferon (Figure 14-10b). After many such interferon-nonresponding mutant cell lines were obtained, they were used to screen a genomic or cDNA library for the wild-type genes that complemented the mutated genes in nonresponding cells, a technique called **functional complementation** (see Figure 9-20). In this case, mutant cells expressing the corresponding recombinant wild-type gene grew on HAT medium and were sensitive to killing by 6-thioguanine in the presence of interferon. That is, they acted like wild-type cells.

Cloning of the genes identified by this procedure led to recognition of two key signal-transduction proteins: a JAK tyrosine kinase and a STAT transcription factor. Subsequent work showed that one (sometimes two) of the four human

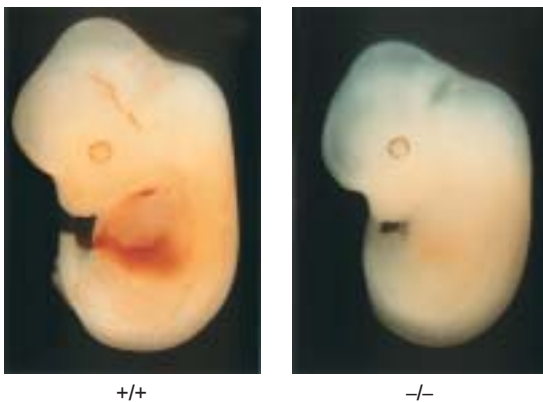
JAK proteins and at least one of several STAT proteins are involved in signaling downstream from all cytokine receptors. To understand how JAK and STAT proteins function, we examine one of the best-understood cytokine signaling pathways, that downstream of the erythropoietin receptor.

Receptor-Associated JAK Kinases Activate STAT Transcription Factors Bound to a Cytokine Receptor

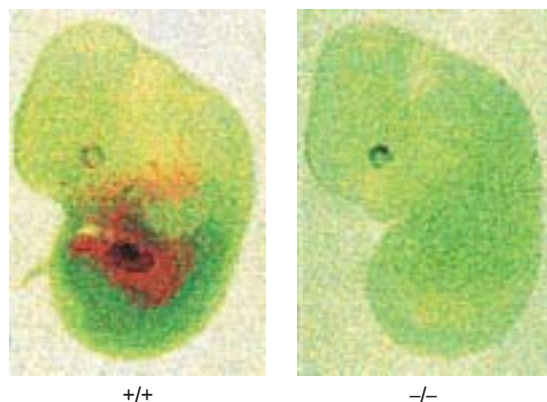
The JAK2 kinase is tightly bound to the cytosolic domain of the erythropoietin receptor (EpoR). Like the three other members of the JAK family of kinases, JAK2 contains an N-terminal receptor-binding domain, a C-terminal kinase domain that is normally poorly active catalytically, and a middle domain of unknown function. JAK2, erythropoietin, and the EpoR are all required for formation of adult-type erythrocytes, which normally begins at day 12 of embryonic development in mice. As Figure 14-11 shows, embryonic mice lacking functional genes encoding either the EpoR or JAK2 cannot form adult-type erythrocytes and eventually die owing to the inability to transport oxygen to the fetal organs.

As already noted, erythropoietin binds simultaneously to the extracellular domains of two EpoR monomers on the cell surface (see Figure 14-8). As a result, the associated JAKs are brought close enough together that one can phosphorylate the other on a critical tyrosine in the activation lip. As with other kinases, phosphorylation of the activation lip leads to a conformational change that reduces the K_m for ATP or the substrate to be phosphorylated, thus increasing the kinase activity. One piece of evidence for this activation mechanism comes from study of a mutant JAK2 in which the critical tyrosine is mutated to phenylalanine. The mutant JAK2 binds normally to the EpoR but cannot be phosphorylated.

EpoR



JAK2



▲ **EXPERIMENTAL FIGURE 14-11 Studies with mutant mice reveal that both the erythropoietin receptor (EpoR) and JAK2 are essential for development of erythrocytes.** Mice in which both alleles of the EpoR or JAK2 gene are “knocked out” develop normally until embryonic day 13, at which time they begin to die of anemia due to the lack of erythrocyte-mediated transport of oxygen to the fetal organs. The red organ in the wild-type embryos (+/+) is the fetal liver, the major site of

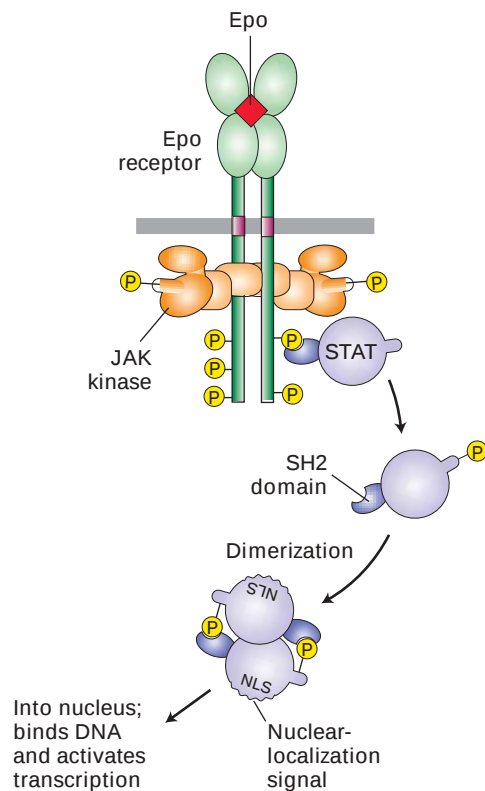
erythrocyte production at this developmental stage. The absence of color in the mutant embryos (–/–) indicates the absence of erythrocytes containing hemoglobin. Otherwise the mutant embryos appear normal, indicating that the main function of the EpoR and JAK2 in early mouse development is to support production of erythrocytes. [EpoR images from H. Wu et al., 1995, *Cell* **83**:59; JAK2 images from H. Neubauer et al., 1998, *Cell* **93**:307]

Expression of this mutant JAK2 in erythroid cells in greater than normal amounts totally blocks EpoR signaling, as the mutant JAK2 blocks the function of the wild-type protein. This type of mutation, referred to as a *dominant negative*, causes loss of function even in cells that carry copies of the wild-type gene (Chapter 9).

Once the JAK kinases become activated, they phosphorylate several tyrosine residues on the cytosolic domain of the receptor. Certain of these phosphotyrosine residues then serve as binding sites for a group of transcription factors collectively termed *STATs*. All STAT proteins contain an N-terminal SH2 domain that binds to a phosphotyrosine in the receptor's cytosolic domain, a central DNA-binding domain, and a C-terminal domain with a critical tyrosine residue. Once a STAT is bound to the receptor, the C-terminal tyro-

sine is phosphorylated by an associated JAK kinase (Figure 14-12). This arrangement ensures that in a particular cell only those STAT proteins with an SH2 domain that can bind to a particular receptor protein will be activated. A phosphorylated STAT dissociates spontaneously from the receptor, and two phosphorylated STAT proteins form a dimer in which the SH2 domain on each binds to the phosphotyrosine in the other. Because dimerization exposes the nuclear-localization signal (NLS), STAT dimers move into the nucleus, where they bind to specific **enhancer** sequences controlling target genes.

Different STATs activate different genes in different cells. In erythroid progenitors, for instance, stimulation by erythropoietin leads to activation of STAT5. The major protein induced by active STAT5 is Bcl-x_L, which prevents the programmed cell death, or **apoptosis**, of these progenitors, allowing them to proliferate and differentiate into erythroid cells (see Figure 14-7). Indeed, mice lacking STAT5 are highly anemic because many of the erythroid progenitors undergo apoptosis even in the presence of high erythropoietin levels. Such mutant mice produce *some* erythrocytes and thus survive, because the erythropoietin receptor is linked to other anti-apoptotic pathways that do not involve STAT proteins (see Figure 14-9).



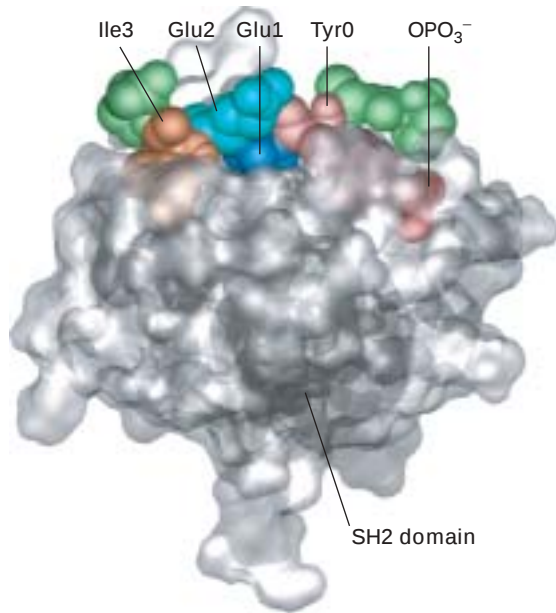
▲ **FIGURE 14-12 JAK-STAT signaling pathway.** Following ligand binding to a cytokine receptor and activation of an associated JAK kinase, JAK phosphorylates several tyrosine residues on the receptor's cytosolic domain (see Figure 14-5, *bottom*). After an inactive monomeric STAT transcription factor binds to a phosphotyrosine in the receptor, it is phosphorylated by active JAK. Phosphorylated STATs spontaneously dissociate from the receptor and spontaneously dimerize. Because the STAT homodimer has two phosphotyrosine-SH2 domain interactions, whereas the receptor-STAT complex is stabilized by only one such interaction, phosphorylated STATs tend not to rebound to the receptor. The STAT dimer, which has two exposed nuclear-localization signals (NLS), moves into the nucleus, where it can bind to promoter sequences and activate transcription of target genes.

SH2 and PTB Domains Bind to Specific Sequences Surrounding Phosphotyrosine Residues

As noted earlier, many intracellular signal-transduction proteins contain an SH2 or PTB domain by which they bind to an activated receptor or other component of a signaling pathway containing a phosphotyrosine residue (see Figure 14-6). The SH2 domain derived its full name, the *Src homology 2 domain*, from its homology with a region in the prototypical cytosolic tyrosine kinase encoded by the *src* gene. The three-dimensional structures of SH2 domains in different proteins are very similar, but each binds to a distinct sequence of amino acids surrounding a phosphotyrosine residue. The unique amino acid sequence of each SH2 domain determines the specific phosphotyrosine residues it binds.

The SH2 domain of the Src tyrosine kinase, for example, binds strongly to any peptide containing a critical four-residue core sequence: phosphotyrosine-glutamic acid-glutamic acid-isoleucine (Figure 14-13). These four amino acids make intimate contact with the peptide-binding site in the Src SH2 domain. Binding resembles the insertion of a two-pronged “plug”—the phosphotyrosine and isoleucine side chains of the peptide—into a two-pronged “socket” in the SH2 domain. The two glutamic acids fit snugly onto the surface of the SH2 domain between the phosphotyrosine socket and the hydrophobic socket that accepts the isoleucine residue.

Variations in the hydrophobic socket in the SH2 domains of different STATs and other signal-transduction proteins allow them to bind to phosphotyrosines adjacent to different sequences, accounting for differences in their binding partners.



▲ **FIGURE 14-13 Surface model of the SH2 domain from Src kinase bound to a phosphotyrosine-containing peptide.** The peptide bound by this SH2 domain (gray) is shown in spacefill. The phosphotyrosine (Tyr0 and OPO_3^- , orange) and isoleucine (Ile3, orange) residues fit into a two-pronged socket on the surface of the SH2 domain; the two glutamate residues (Glu1, dark blue; Glu2, light blue) are bound to sites on the surface of the SH2 domain between the two sockets. Nonbinding residues on the target peptide are colored green. [See G. Waksman et al., 1993, *Cell* **72**:779.]

The binding specificity of SH2 domains is largely determined by residues C-terminal to the phosphotyrosine in a target peptide. In contrast, the binding specificity of PTB domains is determined by specific residues five to eight residues N-terminal to a phosphotyrosine residue. Sometimes a PTB domain binds to a target peptide even if the tyrosine is not phosphorylated.

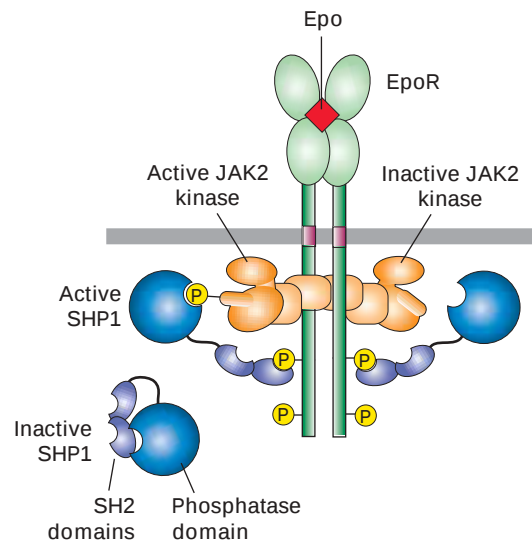
Signaling from Cytokine Receptors Is Modulated by Negative Signals

Signal-induced transcription of target genes for too long a period can be as dangerous for the cell as too little induction. Thus cells must be able to turn off a signaling pathway quickly unless the extracellular signal remains continuously present. In various progenitor cells, two classes of proteins serve to dampen signaling from cytokine receptors, one over the short term (minutes) and the other over longer periods of time.

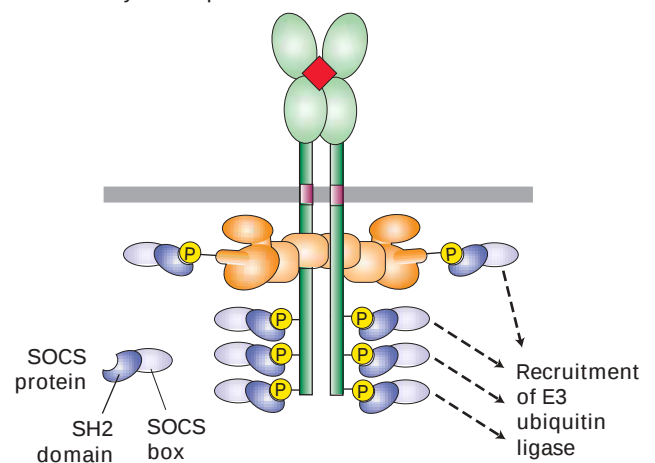
Short-Term Regulation by SHP1 Phosphatase Mutant mice lacking *SHP1 phosphatase* die because of excess production of erythrocytes and several other types of blood cells. Analysis of these mutant mice offered the first suggestion that SHP1, a phosphotyrosine **phosphatase**, negatively regulates signaling from several types of cytokine receptors in several types of progenitor cells.

How SHP1 dampens cytokine signaling is depicted in Figure 14-14a. In addition to a phosphatase catalytic domain, SHP1 has two SH2 domains. When cells are not stimulated

(a) JAK2 deactivation induced by SHP1 phosphatase



(b) Signal blocking and protein degradation induced by SOCS proteins



▲ **FIGURE 14-14 Two mechanisms for terminating signal transduction from the erythropoietin receptor (EpoR).**

(a) SHP1, a protein tyrosine phosphatase, is present in an inactive form in unstimulated cells. Binding of an SH2 domain in SHP1 to a particular phosphotyrosine in the activated receptor unmasks its phosphatase catalytic site and positions it near the phosphorylated tyrosine in the lip region of JAK2. Removal of the phosphate from this tyrosine inactivates the JAK kinase. (b) SOCS proteins, whose expression is induced in erythropoietin-stimulated erythroid cells, inhibit or permanently terminate signaling over longer time periods. Binding of SOCS to phosphotyrosine residues on the EpoR or JAK2 blocks binding of other signaling proteins (*left*). The SOCS box can also target proteins such as JAK2 for degradation by the ubiquitin-proteasome pathway (*right*). Similar mechanisms regulate signaling from other cytokine receptors. [Part (a) adapted from S. Constantinescu et al., 1999, *Trends Endocrin. Metabol.* **10**:18; part (b) adapted from B. T. Kile and W. S. Alexander, 2001, *Cell. Mol. Life Sci.* **58**:1]

by a cytokine (are in the resting state), one of the SH2 domains physically binds to and inactivates the catalytic site in SHP1. In the stimulated state, however, this blocking SH2 domain binds to a specific phosphotyrosine residue in the activated receptor. The conformational change that accompanies this binding unmasks the SHP1 catalytic site and also brings it adjacent to the phosphotyrosine residue in the activation lip of the JAK associated with the receptor. By removing this phosphate, SHP1 inactivates the JAK, so that it can no longer phosphorylate the receptor or other substrates (e.g., STATs) unless additional cytokine molecules bind to cell-surface receptors, initiating a new round of signaling.

Long-Term Regulation by SOCS Proteins Among the genes whose transcription is induced by STAT proteins are those encoding a class of small proteins, termed *SOCS proteins*, that terminate signaling from cytokine receptors. These negative regulators, also known as *CIS proteins*, act in two ways (Figure 14-14b). First, the SH2 domain in several SOCS proteins binds to phosphotyrosines on an activated receptor, preventing binding of other SH2-containing signaling proteins (e.g., STATs) and thus inhibiting receptor signaling. One SOCS protein, SOCS-1, also binds to the critical phosphotyrosine in the activation lip of activated JAK2 kinase, thereby inhibiting its catalytic activity. Second, all SOCS proteins contain a domain, called the *SOCS box*, that recruits components of E3 ubiquitin ligases (see Figure 3-13). As a result of binding SOCS-1, for instance, JAK2 becomes polyubiquitinated and then degraded in **proteasomes**, thus permanently turning off all JAK2-mediated signaling pathways. The observation that proteasome inhibitors prolong JAK2 signal transduction supports this mechanism.

Studies with cultured mammalian cells have shown that the receptor for growth hormone, which belongs to the cytokine receptor superfamily, is down-regulated by another SOCS protein, SOCS-2. Strikingly, mice deficient in this SOCS protein grow significantly larger than their wild-type counterparts and have long bone lengths and proportionate enlargement of most organs. Thus SOCS proteins play an essential negative role in regulating intracellular signaling from the receptors for erythropoietin, growth hormone, and other cytokines.

Mutant Erythropoietin Receptor That Cannot Be Down-Regulated Leads to Increased Hematocrit

In normal adult men and women, the percentage of erythrocytes in the blood (the *hematocrit*) is maintained very close to 45–47 percent. A drop in the hematocrit results in increased production of erythropoietin by the kidney. The elevated erythropoietin level causes more erythroid progenitors to undergo terminal proliferation and differentiation into mature erythrocytes, soon restoring the hematocrit to its normal level. In endurance sports, such as cross-country skiing, where oxygen transport to the muscles may become limiting,

an excess of red blood cells may confer a competitive advantage. For this reason, use of supplemental erythropoietin to increase the hematocrit above the normal level is banned in many athletic competitions, and athletes are regularly tested for the presence of commercial recombinant erythropoietin in their blood and urine.



Supplemental erythropoietin not only confers a possible competitive advantage but also can be dangerous. Too many red cells can cause the blood to become sluggish and clot in small blood vessels, especially in the brain. Several athletes who doped themselves with erythropoietin have died of a stroke while exercising.

Discovery of a mutant, unregulated erythropoietin receptor (EpoR) explained a suspicious situation in which a winner of three gold medals in Olympic cross-country skiing was found to have a hematocrit above 60 percent. Testing for erythropoietin in his blood and urine, however, revealed lower-than-normal amounts. Subsequent DNA analysis showed that the athlete was heterozygous for a mutation in the gene encoding the erythropoietin receptor. The mutant allele encoded a truncated receptor missing several of the tyrosines that normally become phosphorylated after stimulation by erythropoietin. As a consequence, the mutant receptor was able to activate STAT5 and other signaling proteins normally, but was unable to bind the negatively acting SHP1 phosphatase, which usually terminates signaling (see Figure 14-14a). Thus the very low level of erythropoietin produced by this athlete induced prolonged intracellular signaling in his erythroid progenitor cells, resulting in production of higher-than-normal numbers of erythrocytes. This example vividly illustrates the fine level of control over signaling from the erythropoietin receptor in the human body. ■

KEY CONCEPTS OF SECTION 14.2

Cytokine Receptors and the JAK-STAT Pathway

- Two receptor classes, cytokine receptors and receptor tyrosine kinases, transduce signals via their associated or intrinsic protein tyrosine kinases. Ligand binding triggers formation of functional dimeric receptors and phosphorylation of the activation lip in the kinases, enhancing their catalytic activity (see Figure 14-5).
- All cytokines are constructed of four α helices that are folded in a characteristic arrangement.
- Erythropoietin, a cytokine secreted by kidney cells, prevents apoptosis and promotes proliferation and differentiation of erythroid progenitor cells in the bone marrow. An excess of erythropoietin or mutations in its receptor that prevent down-regulation result in production of elevated numbers of red blood cells.
- All cytokine receptors are closely associated with a JAK protein tyrosine kinase, which can activate several down-

stream signaling pathways leading to changes in transcription of target genes or in the activity of proteins that do not regulate transcription (see Figure 14-9).

- The JAK-STAT pathway operates downstream of all cytokine receptors. STAT monomers bound to receptors are phosphorylated by receptor-associated JAKs, then dimerize and move to the nucleus, where they activate transcription (see Figure 14-12).
- Short peptide sequences containing phosphotyrosine residues are bound by SH2 and PTB domains, which are found in many signal-transducing proteins. Such protein-protein interactions are important in many signaling pathways.
- Signaling from cytokine receptors is terminated by the phosphotyrosine phosphatase SHP1 and several SOCS proteins (see Figure 14-14).

14.3 Receptor Tyrosine Kinases and Activation of Ras

We return now to the receptor tyrosine kinases (RTKs), which have intrinsic protein tyrosine kinase activity in their cytosolic domains. The ligands for RTKs are soluble or membrane-bound peptide or protein hormones including nerve growth factor (NGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and insulin. Ligand-induced activation of an RTK stimulates its tyrosine kinase activity, which subsequently stimulates the *Ras*–*MAP kinase* pathway and several other signal-transduction pathways. RTK signaling pathways have a wide spectrum of functions including regulation of cell proliferation and differentiation, promotion of cell survival, and modulation of cellular metabolism.



Some RTKs have been identified in studies on human cancers associated with mutant forms of growth-factor receptors, which send a proliferative signal to cells even in the absence of growth factor. For example, a constitutively active mutant form of Her2, a receptor for EGF-like proteins, enables uncontrolled proliferation of cancer cells even in the absence of EGF, which is required for proliferation of normal cells (see Figure 23-14). Alternatively, overproduction of the wild-type receptor for EGF in certain human breast cancers results in proliferation at low EGF levels that do not stimulate normal cells; monoclonal antibodies targeted to the EGF receptor have proved therapeutically useful in these patients. Other RTKs have been uncovered during analysis of developmental mutations that lead to blocks in differentiation of certain cell types in *C. elegans*, *Drosophila*, and the mouse. ■

Here we discuss how ligand binding leads to activation of RTKs and how activated receptors transmit a signal to the

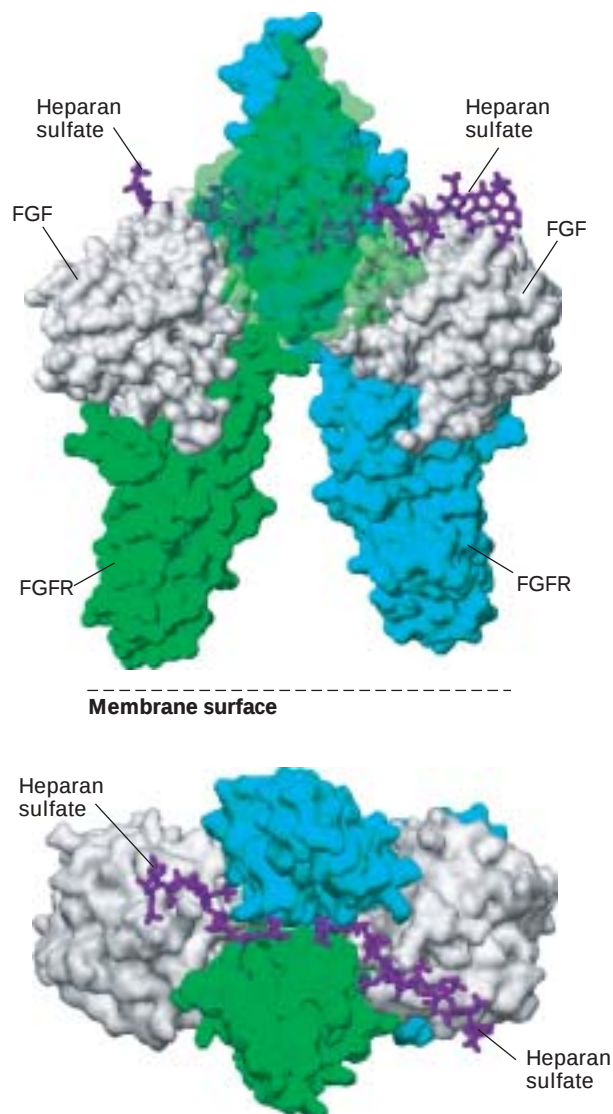
Ras protein, the GTPase switch protein that functions in transducing signals from many different RTKs. The transduction of signals downstream from Ras to a common cascade of serine/threonine kinases, leading ultimately to activation of MAP kinase and certain transcription factors, is covered in the following section.

Ligand Binding Leads to Transphosphorylation of Receptor Tyrosine Kinases

All RTKs constitute an extracellular domain containing a ligand-binding site, a single hydrophobic transmembrane α helix, and a cytosolic domain that includes a region with protein tyrosine kinase activity. Most RTKs are monomeric, and ligand binding to the extracellular domain induces formation of receptor dimers, as depicted in Figure 14-4 for the EGF receptor. Some monomeric ligands, including FGF, bind tightly to heparan sulfate, a negatively charged polysaccharide component of the extracellular matrix (Chapter 6); this association enhances ligand binding to the monomeric receptor and formation of a dimeric receptor-ligand complex (Figure 14-15). The ligands for some RTKs are dimeric; their binding brings two receptor monomers together directly. Yet other RTKs, such as the insulin receptor, form disulfide-linked dimers in the absence of hormone; binding of ligand to this type of RTK alters its conformation in such a way that the receptor becomes activated.

Regardless of the mechanism by which ligand binds and locks an RTK into a functional dimeric state, the next step is universal. In the resting, unstimulated state, the intrinsic kinase activity of an RTK is very low. In the dimeric receptor, however, the kinase in one subunit can phosphorylate one or more tyrosine residues in the activation lip near the catalytic site in the other subunit. This leads to a conformational change that facilitates binding of ATP in some receptors (e.g., insulin receptor) and binding of protein substrates in other receptors (e.g., FGF receptor). The resulting enhanced kinase activity then phosphorylates other sites in the cytosolic domain of the receptor. This ligand-induced activation of RTK kinase activity is analogous to the activation of the JAK kinases associated with cytokine receptors (see Figure 14-5). The difference resides in the location of the kinase catalytic site, which is within the cytosolic domain of RTKs, but within a separate JAK kinase in the case of cytokine receptors.

As in signaling by cytokine receptors, phosphotyrosine residues in activated RTKs serve as docking sites for proteins involved in downstream signal transduction. Many phosphotyrosine residues in activated RTKs interact with *adapter proteins*, small proteins that contain SH2, PTB, or SH3 domains but have no intrinsic enzymatic or signaling activities (see Figure 14-6). These proteins couple activated RTKs to other components of signal-transduction pathways such as the one involving Ras activation.



▲ FIGURE 14-15 Structure of the dimerized ligand-bound receptor for fibroblast growth factor (FGF), which is stabilized by heparan sulfate. Shown here are side and top views of the complex comprising the extracellular domains of two FGF receptor (FGFR) monomers (green and blue), two bound FGF molecules (white), and two short heparan sulfate chains (purple), which bind tightly to FGF. In the side view, the upper domain of one receptor (blue) is situated behind that of the other (green). In the top view, the heparan sulfate chains thread between and make numerous contacts with the upper domains of both receptor monomers. These interactions promote binding of the ligand to the receptor and receptor dimerization.

[Adapted from J. Schlessinger et al., 2000, *Mol. Cell* **6**:743.]

Ras, a GTPase Switch Protein, Cycles Between Active and Inactive States

Ras is a monomeric GTP-binding switch protein that, like the G_{α} subunits in trimeric G proteins, alternates between an active *on* state with a bound GTP and an inactive *off* state

with a bound GDP. As discussed in Chapter 13, trimeric G proteins are directly linked to cell-surface receptors and transduce signals, via the G_{α} subunit, to various effectors such as adenylyl cyclase. In contrast, Ras is not directly linked to cell-surface receptors.

Ras activation is accelerated by a *guanine nucleotide-exchange factor (GEF)*, which binds to the Ras·GDP complex, causing dissociation of the bound GDP (see Figure 3-29). Because GTP is present in cells at a higher concentration than GDP, GTP binds spontaneously to “empty” Ras molecules, with release of GEF and formation of the active Ras·GTP. Subsequent hydrolysis of the bound GTP to GDP deactivates Ras. Unlike the deactivation of G_{α} ·GTP, deactivation of Ras·GTP requires the assistance of another protein, a *GTPase-activating protein (GAP)* that binds to Ras·GTP and accelerates its intrinsic GTPase activity by more than a hundredfold. Thus the average lifetime of a GTP bound to Ras is about 1 minute, which is much longer than the average lifetime of G_{α} ·GTP. In cells, GAP binds to specific phosphotyrosines in activated RTKs, bringing it close enough to membrane-bound Ras·GTP to exert its accelerating effect on GTP hydrolysis. The actual hydrolysis of GTP is catalyzed by amino acids from both Ras and GAP. In particular, insertion of an arginine side chain on GAP into the Ras active site stabilizes an intermediate in the hydrolysis reaction.

The differences in the cycling mechanisms of Ras and G_{α} are reflected in their structures. Ras (≈ 170 amino acids) is smaller than G_{α} proteins (≈ 300 amino acids), but its three-dimensional structure is similar to that of the GTPase domain of G_{α} (see Figure 13-8). Recent structural and biochemical studies show that G_{α} also contains another domain that apparently functions like GAP to increase the rate of GTP hydrolysis by G_{α} . In addition, the direct interaction between an activated receptor and inactive G protein promotes release of GDP and binding of GTP, so that a separate nucleotide exchange factor is not required.

Both the trimeric G proteins and Ras are members of a family of intracellular GTP-binding switch proteins collectively referred to as the **GTPase superfamily**, which we introduced in Chapter 3. The many similarities between the structure and function of Ras and G_{α} and the identification of both proteins in all eukaryotic cells indicate that a single type of signal-transducing GTPase originated very early in evolution. In fact, their structures are similar to those of the GTP-binding factors involved in protein synthesis, which are found in all prokaryotic and eukaryotic cells. The gene encoding this ancestral protein subsequently duplicated and evolved to the extent that the human genome encodes a superfamily of such GTPases, comprising perhaps a hundred different intracellular switch proteins. These related proteins control many aspects of cellular growth and metabolism.



Mammalian Ras proteins have been studied in great detail because mutant Ras proteins are associated with many types of human cancer. These mutant proteins, which bind but cannot hydrolyze GTP, are

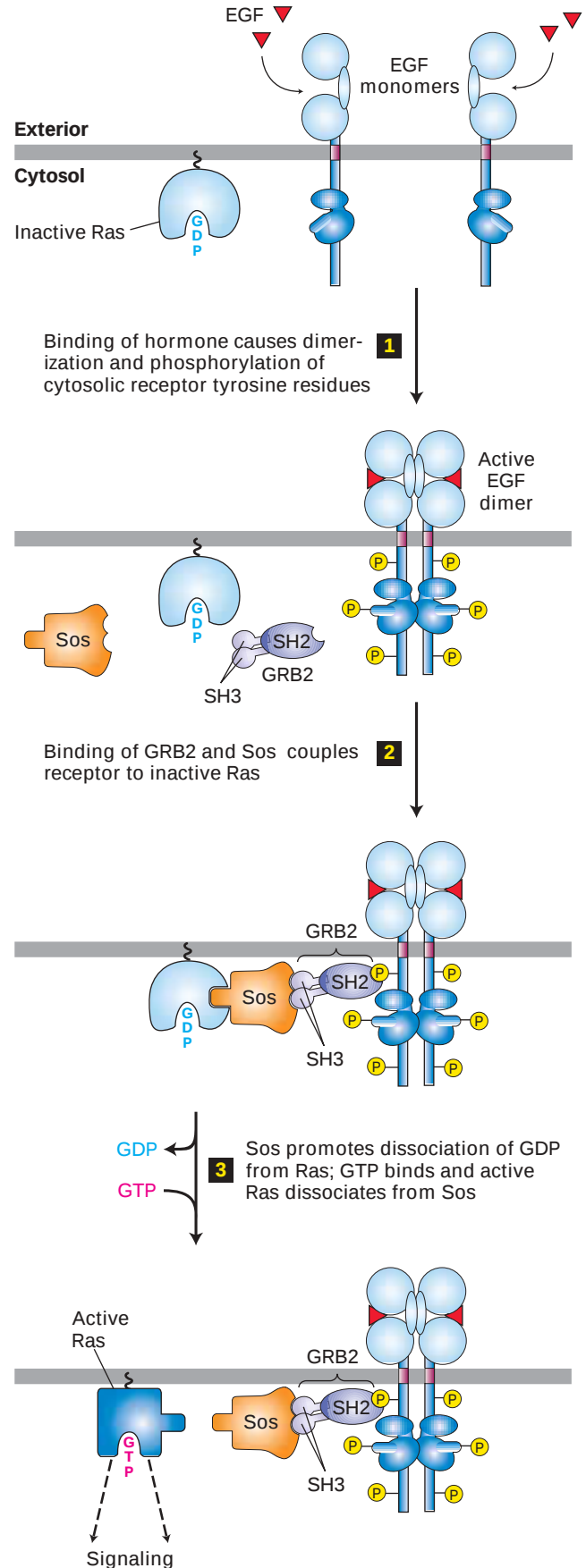
permanently in the “on” state and contribute to neoplastic **transformation** (Chapter 23). Determination of the three-dimensional structure of the Ras-GAP complex explained the puzzling observation that most oncogenic, constitutively active Ras proteins (Ras^D) contain a mutation at position 12. Replacement of the normal glycine-12 with any other amino acid (except proline) blocks the functional binding of GAP, and in essence “locks” Ras in the active GTP-bound state. ■

An Adapter Protein and Guanine Nucleotide–Exchange Factor Link Most Activated Receptor Tyrosine Kinases to Ras

The first indication that Ras functions downstream from RTKs in a common signaling pathway came from experiments in which cultured fibroblast cells were induced to proliferate by treatment with a mixture of PDGF and EGF. Microinjection of anti-Ras antibodies into these cells blocked cell proliferation. Conversely, injection of Ras^D, a constitutively active mutant Ras protein that hydrolyzes GTP very inefficiently and thus persists in the active state, caused the cells to proliferate in the absence of the growth factors. These findings are consistent with studies showing that addition of FGF to fibroblasts leads to a rapid increase in the proportion of Ras present in the GTP-bound active form.

How does binding of a growth factor (e.g., EGF) to an RTK (e.g., the EGF receptor) lead to activation of Ras? Two cytosolic proteins—GRB2 and Sos—provide the key links (Figure 14-16). An SH2 domain in GRB2 binds to a specific phosphotyrosine residue in the activated receptor. GRB2 also contains two SH3 domains, which bind to and activate Sos. GRB2 thus functions as an adapter protein for the EGF receptor. Sos is a guanine nucleotide–exchange protein (GEF), which catalyzes conversion of inactive GDP-bound Ras to the active GTP-bound form. Genetic analyses of mutants in the worm *C. elegans* and in the fly *Drosophila* blocked at particular stages of differentiation were critical in elucidating the roles of these two proteins in linking an RTK to Ras activation. To illustrate the power of this experimental approach, we consider development of a particular type of cell in the compound eye of *Drosophila*.

► **FIGURE 14-16 Activation of Ras following ligand binding to receptor tyrosine kinases (RTKs).** The receptors for epidermal growth factor (EGF) and many other growth factors are RTKs. The cytosolic adapter protein GRB2 binds to a specific phosphotyrosine on an activated, ligand-bound receptor and to the cytosolic Sos protein, bringing it near its substrate, the inactive Ras·GDP. The guanine nucleotide–exchange factor (GEF) activity of Sos then promotes formation of active Ras·GTP. Note that Ras is tethered to the membrane by a hydrophobic farnesyl anchor (see Figure 5-15). [See J. Schlessinger, 2000, *Cell* **103**:211, and M. A. Simon, 2000, *Cell* **103**:13.]



Genetic Studies in *Drosophila* Identify Key Signal-Transducing Proteins Downstream from Receptor Tyrosine Kinases

The compound eye of the fly is composed of some 800 individual eyes called *ommatidia* (Figure 14-17a). Each ommatidium consists of 22 cells, eight of which are photosensitive neurons called *retinula*, or R cells, designated R1–R8 (Figure 14-17b). An RTK called *Sevenless* (*Sev*) specifically regulates development of the R7 cell and is not essential for any other known function. In flies with a mutant *sevenless* (*sev*) gene, the R7 cell in each ommatidium does not form (Figure 14-17c). Since the R7 photoreceptor is necessary for flies to see in ultraviolet light, mutants that lack functional R7 cells but are otherwise normal are easily isolated.

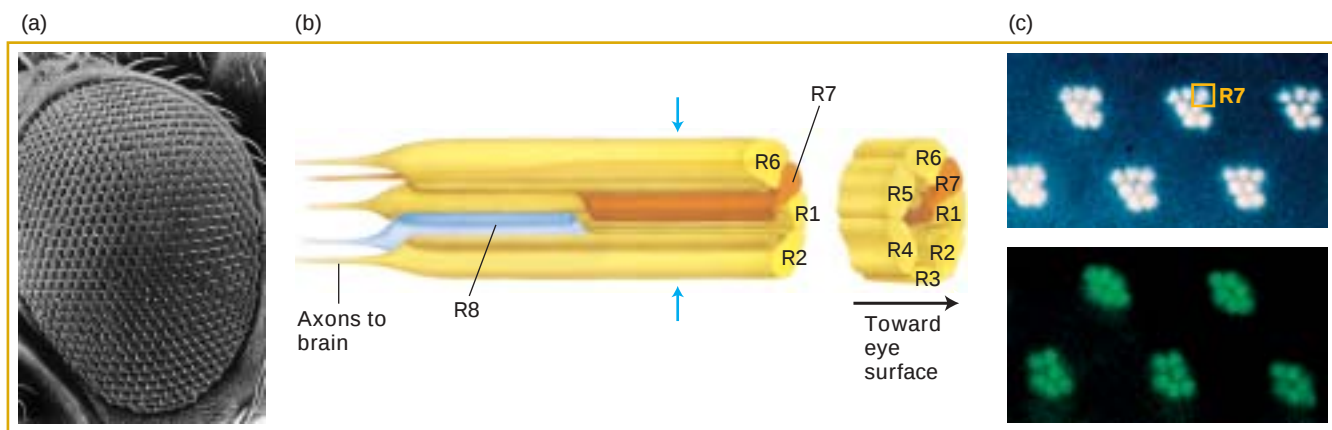
During development of each ommatidium, a protein called *Boss* (*Bride of Sevenless*) is expressed on the surface of the R8 cell. This membrane-tethered protein is the ligand for the *Sev* RTK on the surface of the neighboring R7 precursor cell, signaling it to develop into a photosensitive neuron (Figure 14-18a). In mutant flies that do not express a functional *Boss* protein or *Sev* RTK, interaction between the *Boss* and *Sev* proteins cannot occur, and no R7 cells develop (Figure 14-18b).

To identify intracellular signal-transducing proteins in the *Sev* RTK pathway, investigators produced mutant flies expressing a temperature-sensitive *Sev* protein. When these flies were maintained at a permissive temperature, all their ommatidia contained R7 cells; when they were maintained at a nonpermissive temperature, no R7 cells developed. At a par-

ticular intermediate temperature, however, just enough of the *Sev* RTK was functional to mediate normal R7 development. The investigators reasoned that at this intermediate temperature, the signaling pathway would become defective (and thus no R7 cells would develop) if the level of *another* protein involved in the pathway was reduced, thus reducing the activity of the overall pathway below the level required to form an R7 cell. A recessive mutation affecting such a protein would have this effect because, in diploid organisms like *Drosophila*, a heterozygote containing one wild-type and one mutant allele of a gene will produce half the normal amount of the gene product; hence, even if such a recessive mutation is in an essential gene, the organism will be viable. However, a fly carrying a temperature-sensitive mutation in the *sev* gene and a second mutation affecting another protein in the signaling pathway would be expected to lack R7 cells at the intermediate temperature.

By use of this screen, researchers identified the genes encoding three important proteins in the *Sev* pathway (see Figure 14-16): an SH2-containing adapter protein exhibiting 64 percent identity to human GRB2; a guanine nucleotide-exchange factor called *Sos* (Son of Sevenless) exhibiting 45 percent identity with its mouse counterpart; and a Ras protein exhibiting 80 percent identity with its mammalian counterparts. These three proteins later were found to function in other signaling pathways initiated by ligand binding to different RTK receptors and used at different times and places in the developing fly.

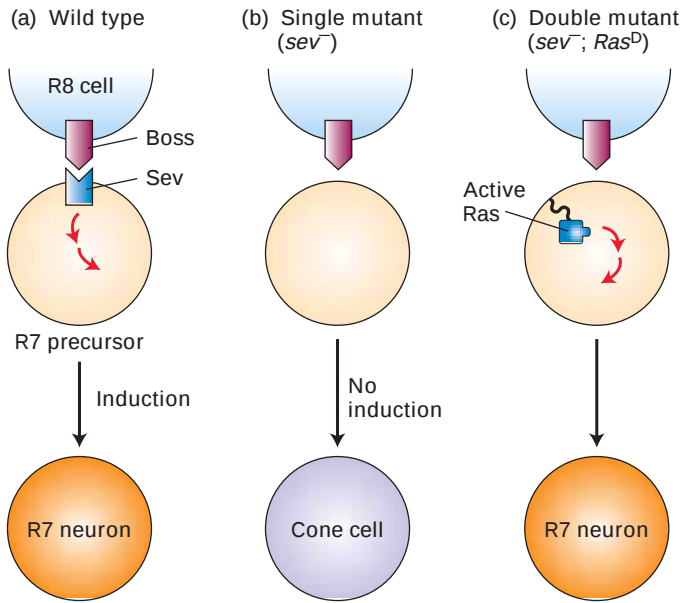
In subsequent studies, researchers introduced a mutant *ras^D* gene into fly embryos carrying the *sevenless* mutation.



▲ FIGURE 14-17 The compound eye of *Drosophila melanogaster*. (a) Scanning electron micrograph showing individual ommatidia that compose the fruit fly eye.

(b) Longitudinal and cutaway views of a single ommatidium. Each of these tubular structures contains eight photoreceptors, designated R1–R8, which are long, cylindrically shaped light-sensitive cells. R1–R6 (yellow) extend throughout the depth of the retina, whereas R7 (brown) is located toward the surface of the eye, and R8 (blue) toward the backside, where the axons exit. (c) Comparison of eyes from wild-type and *sevenless*

mutant flies viewed by a special technique that can distinguish the photoreceptors in an ommatidium. The plane of sectioning is indicated by the blue arrows in (b), and the R8 cell is out of the plane of these images. The seven photoreceptors in this plane are easily seen in the wild-type ommatidia (*top*), whereas only six are visible in the mutant ommatidia (*bottom*). Flies with the *sevenless* mutation lack the R7 cell in their eyes. [Part (a) from E. Hafen and K. Basler, 1991, *Development* 1 (suppl.):123; part (b) adapted from R. Reinke and S. L. Zipursky, 1988, *Cell* 55:321; part (c) courtesy of U. Banerjee.]



▲ **EXPERIMENTAL FIGURE 14-18 Genetic studies reveal that activation of Ras induces development of R7 photoreceptors in the *Drosophila* eye.** (a) During larval development of wild-type flies, the R8 cell in each developing ommatidium expresses a cell-surface protein, called Boss, that binds to the Sev RTK on the surface of its neighboring R7 precursor cell. This interaction induces changes in gene expression that result in differentiation of the precursor cell into a functional R7 neuron. (b) In fly embryos with a mutation in the *sevenless* (*sev*) gene, R7 precursor cells cannot bind Boss and therefore do not differentiate normally into R7 cells. Rather the precursor cell enters an alternative developmental pathway and eventually becomes a cone cell. (c) Double-mutant larvae (*sev*⁻; *Ras*^D) express a constitutively active Ras (*Ras*^D) in the R7 precursor cell, which induces differentiation of R7 precursor cells in the absence of the Boss-mediated signal. This finding shows that activated Ras is sufficient to mediate induction of an R7 cell. [See M. A. Simon et al., 1991, *Cell* **67**:701, and M. E. Fortini et al., 1992, *Nature* **355**:559.]

As noted earlier, the *ras*^D gene encodes a constitutive Ras protein that is present in the active GTP-bound form even in the absence of a hormone signal. Although no functional Sev RTK was expressed in these double-mutants (*sev*⁻; *ras*^D), R7 cells formed normally, indicating that activation of Ras is sufficient for induction of R7-cell development (Figure 14-18c). This finding, which is consistent with the results with cultured fibroblasts described earlier, supports the conclusion that activation of Ras is a principal step in intracellular signaling by most if not all RTKs.

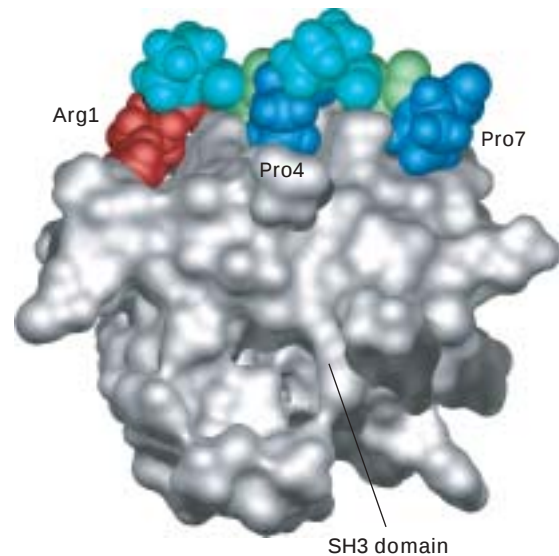
Binding of Sos Protein to Inactive Ras Causes a Conformational Change That Activates Ras

The adapter protein GRB2 contains two SH3 domains, which bind to Sos, a guanine nucleotide-exchange factor, in

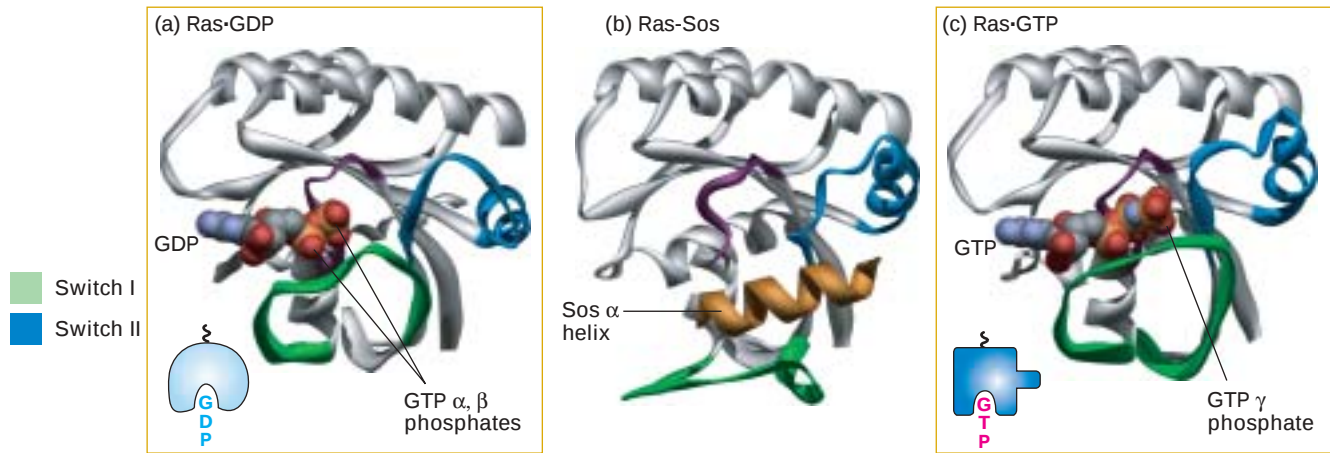
addition to an SH2 domain, which binds to phosphotyrosine residues in RTKs. Like phosphotyrosine-binding SH2 and PTB domains, SH3 domains are present in a large number of proteins involved in intracellular signaling. Although the three-dimensional structures of various SH3 domains are similar, their specific amino acid sequences differ. The SH3 domains in GRB2 selectively bind to proline-rich sequences in Sos; different SH3 domains in other proteins bind to proline-rich sequences distinct from those in Sos.

Proline residues play two roles in the interaction between an SH3 domain in an adapter protein (e.g., GRB2) and a proline-rich sequence in another protein (e.g., Sos). First, the proline-rich sequence assumes an extended conformation that permits extensive contacts with the SH3 domain, thereby facilitating interaction. Second, a subset of these prolines fit into binding “pockets” on the surface of the SH3 domain (Figure 14-19). Several nonproline residues also interact with the SH3 domain and are responsible for determining the binding specificity. Hence the binding of proteins to SH3 and to SH2 domains follows a similar strategy: certain residues provide the overall structural motif necessary for binding, and neighboring residues confer specificity to the binding.

Following activation of an RTK (e.g., Sevenless or the EGF receptor), a complex containing the activated receptor, GRB2, and Sos is formed on the cytosolic face of the plasma membrane (see Figure 14-16). Formation of this complex depends on the ability of GRB2 to bind simultaneously to the receptor and to Sos. Thus receptor activation leads to



▲ **FIGURE 14-19 Surface model of an SH3 domain bound to a short, proline-rich target peptide.** The target peptide is shown as a space-filling model. In this target peptide, two prolines (Pro4 and Pro7, dark blue) fit into binding pockets on the surface of the SH3 domain. Interactions involving an arginine (Arg1, red), two other prolines (light blue), and other residues in the target peptide (green) determine the specificity of binding. [After H. Yu et al., 1994, *Cell* **76**:933.]



▲ FIGURE 14-20 Structures of Ras bound to GDP, Sos protein, and GTP. (a) In Ras·GDP, the Switch I and Switch II segments do not directly interact with GDP (see Figure 13-8). (b) One α helix (orange) in Sos binds to both switch regions of Ras·GDP, leading to a massive conformational change in Ras. In effect, Sos pries Ras open by displacing the Switch I region, thereby allowing GDP to diffuse out. (c) GTP is thought to bind to

Ras·Sos first through its base; subsequent binding of the GTP phosphates completes the interaction. The resulting conformational change in Switch I and Switch II segments of Ras, allowing both to bind to the GTP γ phosphate (see Figure 13-8), displaces Sos and promotes interaction of Ras·GTP with its effectors (discussed later). [Adapted from P. A. Boriack-Sjodin and J. Kuriyan, 1998, *Nature* **394**:341]

relocalization of Sos from the cytosol to the membrane, bringing Sos near to its substrate, namely, membrane-bound Ras·GDP. Biochemical and genetic studies indicate that the C-terminus of Sos inhibits its nucleotide-exchange activity and that GRB2 binding relieves this inhibition.

Binding of Sos to Ras·GDP leads to conformational changes in the Switch I and Switch II segments of Ras, thereby opening the binding pocket for GDP so it can diffuse out (Figure 14-20). Because GTP is present in cells at a concentration some 10 times higher than GDP, GTP binding occurs preferentially, leading to activation of Ras. The activation of Ras and G_{α} thus occurs by similar mechanisms: a conformational change induced by binding of a protein—Sos and an activated G protein-coupled receptor, respectively—that opens the protein structure so bound GDP is released to be replaced by GTP. Binding of GTP to Ras, in turn, induces a specific conformation of Switch I and Switch II that allows Ras·GTP to activate downstream effector molecules, as we discuss in the next section.

KEY CONCEPTS OF SECTION 14.3

Receptor Tyrosine Kinases and Activation of Ras

- Receptor tyrosine kinases (RTKs), which bind to peptide and protein hormones, may exist as preformed dimers or dimerize during binding to ligands.
- Ligand binding leads to activation of the intrinsic protein tyrosine kinase activity of the receptor and phosphorylation of tyrosine residues in its cytosolic domain (see Figure 14-5, *top*). The activated receptor also can phosphorylate other protein substrates.

- Ras is an intracellular GTPase switch protein that acts downstream from most RTKs. Like G_{α} , Ras cycles between an inactive GDP-bound form and an active GTP-bound form. Ras cycling requires the assistance of two proteins, a guanine nucleotide-exchange factor (GEF) and a GTPase-activating protein (GAP).

- RTKs are linked indirectly to Ras via two proteins: GRB2, an adapter protein, and Sos, which has GEF activity (see Figure 14-16).

- The SH2 domain in GRB2 binds to a phosphotyrosine in activated RTKs, while its two SH3 domains bind Sos, thereby bringing Sos close to membrane-bound Ras·GDP and activating its nucleotide exchange activity.

- Binding of Sos to inactive Ras causes a large conformational change that permits release of GDP and binding of GTP, forming active Ras (see Figure 14-20). GAP, which accelerates GTP hydrolysis, is localized near Ras·GTP by binding to activated RTKs.

- Normally, Ras activation and the subsequent cellular response require ligand binding to an RTK or a cytokine receptor. In cells that contain a constitutively active Ras, the cellular response occurs in the absence of ligand binding.

14.4 MAP Kinase Pathways

In mammalian cells all receptor tyrosine kinases (RTKs), as well as most cytokine receptors, appear to utilize a highly conserved signal-transduction pathway in which the signal induced by ligand binding is carried via GRB2 and Sos to Ras, leading to its activation (see Figure 14-16). Activated Ras pro-

motes formation at the membrane of signaling complexes containing three sequentially acting protein kinases that are associated with a scaffold protein. This *kinase cascade* culminates in activation of **MAP kinase**, a serine/threonine kinase also known as *ERK*. After translocating into the nucleus, MAP kinase can phosphorylate many different proteins, including transcription factors that regulate expression of important cell-cycle and differentiation-specific proteins. Activation of MAP kinase in two different cells can lead to similar or different cellular responses, as can its activation in the same cell following stimulation by different hormones.

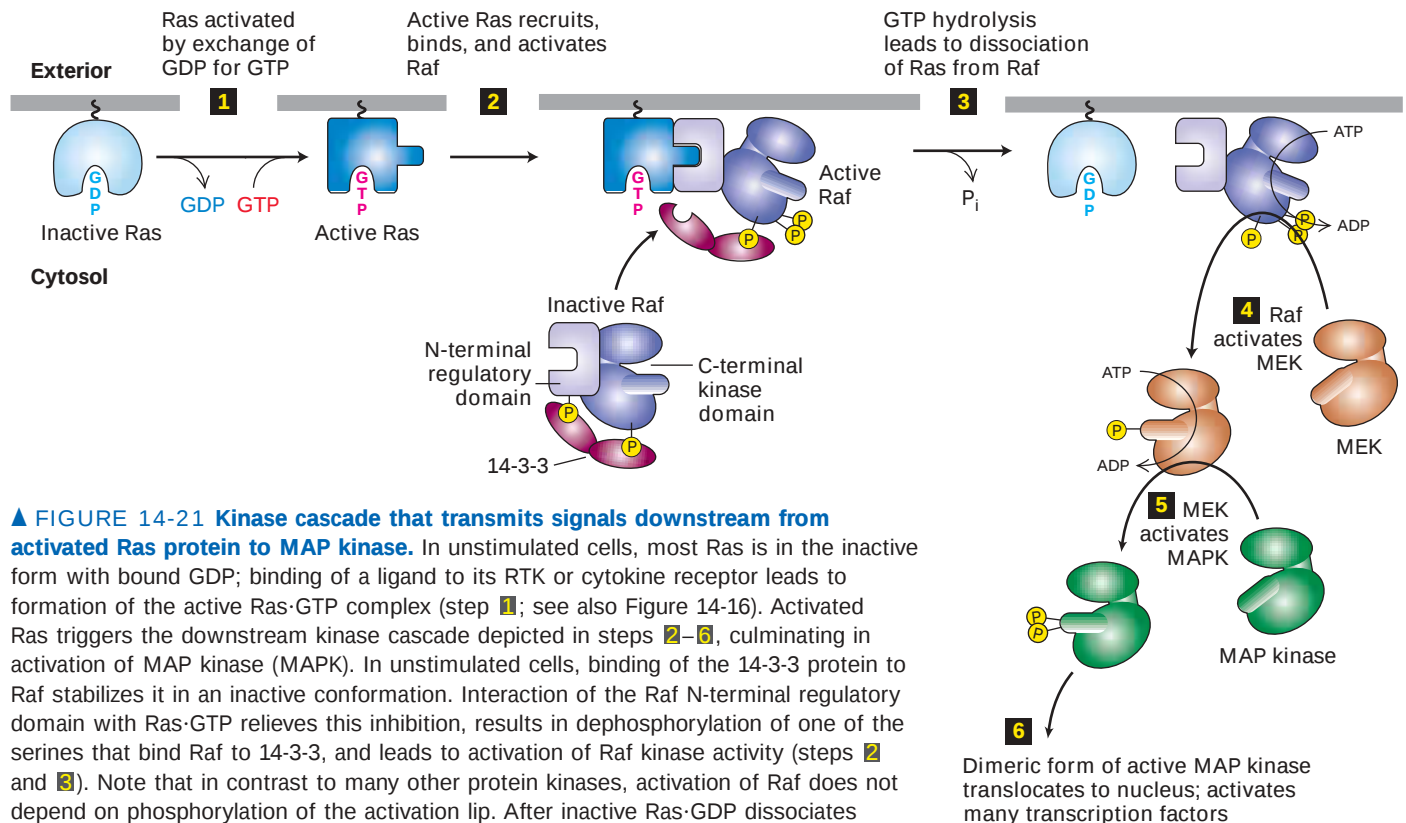
In this section, we first examine the components of the kinase cascade downstream from Ras in RTK-Ras signaling pathways in mammalian cells. Then we discuss the linkage of other signaling pathways to similar kinase cascades, and we examine recent studies indicating that both yeasts and cells of higher eukaryotes contain multiple MAP kinase pathways.

Signals Pass from Activated Ras to a Cascade of Protein Kinases

A remarkable convergence of biochemical and genetic studies in yeast, *C. elegans*, *Drosophila*, and mammals has revealed a highly conserved cascade of protein kinases that operates

in sequential fashion downstream from activated Ras (Figure 14-21). Active Ras·GTP binds to the N-terminal regulatory domain of *Raf*, a serine/threonine kinase, thereby activating it (step [2]). Hydrolysis of Ras·GTP to Ras·GDP releases active Raf (step [3]), which phosphorylates and thereby activates *MEK* (step [4]). Active MEK then phosphorylates and activates MAP kinase, another serine/threonine kinase (step [5]). (A dual-specificity protein kinase, MEK phosphorylates its target proteins on both tyrosine and serine or threonine residues.) MAP kinase phosphorylates many different proteins, including nuclear transcription factors, that mediate cellular responses (step [6]).

Several types of experiments have demonstrated that Raf, MEK, and MAP kinase lie downstream from Ras and have revealed the sequential order of these proteins in the pathway. For example, mutant Raf proteins missing the N-terminal regulatory domain are constitutively active and induce quiescent cultured cells to proliferate in the absence of stimulation by growth factors. These mutant Raf proteins were initially identified in tumor cells; like the constitutively active Ras^D protein, such mutant Raf proteins are said to be encoded by **oncogenes** (Chapter 23). Conversely, cultured mammalian cells that express a mutant, nonfunctional Raf protein cannot be stimulated to proliferate uncontrollably by a constitutively active Ras^D protein. This finding established



▲ FIGURE 14-21 Kinase cascade that transmits signals downstream from activated Ras protein to MAP kinase. In unstimulated cells, most Ras is in the inactive form with bound GDP; binding of a ligand to its RTK or cytokine receptor leads to formation of the active Ras·GTP complex (step [1]; see also Figure 14-16). Activated Ras triggers the downstream kinase cascade depicted in steps [2]–[6], culminating in activation of MAP kinase (MAPK). In unstimulated cells, binding of the 14-3-3 protein to Raf stabilizes it in an inactive conformation. Interaction of the Raf N-terminal regulatory domain with Ras·GTP relieves this inhibition, results in dephosphorylation of one of the serines that bind Raf to 14-3-3, and leads to activation of Raf kinase activity (steps [2] and [3]). Note that in contrast to many other protein kinases, activation of Raf does not depend on phosphorylation of the activation lip. After inactive Ras·GDP dissociates from Raf, it presumably can be reactivated by signals from activated receptors, thereby recruiting additional Raf molecules to the membrane. See the text for details. [See E. Kerkhoff and U. Rapp, 2001, *Adv. Enzyme Regul.* **41**:261; J. Avruch et al., 2001, *Recent Prog. Hormone Res.* **56**:127; and M. Yip-Schneider et al., 2000, *Biochem. J.* **351**:151]

a link between the Raf and Ras proteins. In vitro binding studies further showed that the purified Ras·GTP complex binds directly to the N-terminal regulatory domain of Raf and activates its catalytic activity. An interaction between the mammalian Ras and Raf proteins also was demonstrated in the *yeast two-hybrid system*, a genetic system in yeast used to select cDNAs encoding proteins that bind to target, or “bait,” proteins (see Figure 11-39).

That MAP kinase is activated in response to Ras activation was demonstrated in quiescent cultured cells expressing a constitutively active Ras^D protein. In these cells activated MAP kinase is generated in the absence of stimulation by growth-promoting hormones. More importantly, R7 photoreceptors develop normally in the developing eye of *Drosophila* mutants that lack a functional Ras or Raf protein but express a constitutively active MAP kinase. This finding indicates that activation of MAP kinase is sufficient to transmit a proliferation or differentiation signal normally initiated by ligand binding to a receptor tyrosine kinase such as Sevenless (see Figure 14-18). Biochemical studies showed, however, that Raf cannot directly phosphorylate MAP kinase or otherwise activate its activity.

The final link in the kinase cascade activated by Ras·GTP emerged from studies in which scientists fractionated extracts of cultured cells searching for a kinase activity that could phosphorylate MAP kinase and that was present only in cells stimulated with growth factors, not quiescent cells. This work led to identification of MEK, a kinase that specifically phosphorylates one threonine and one tyrosine residue on MAP kinase, thereby activating its catalytic activity. (The acronym MEK comes from MAP and ERK kinase.) Later studies showed that MEK binds to the C-terminal catalytic domain of Raf and is phosphorylated by the Raf serine/threonine kinase; this phosphorylation activates the catalytic activity of MEK. Hence, activation of Ras induces a kinase cascade that includes Raf, MEK, and MAP kinase: activated RTK → Ras → Raf → MEK → MAP kinase.

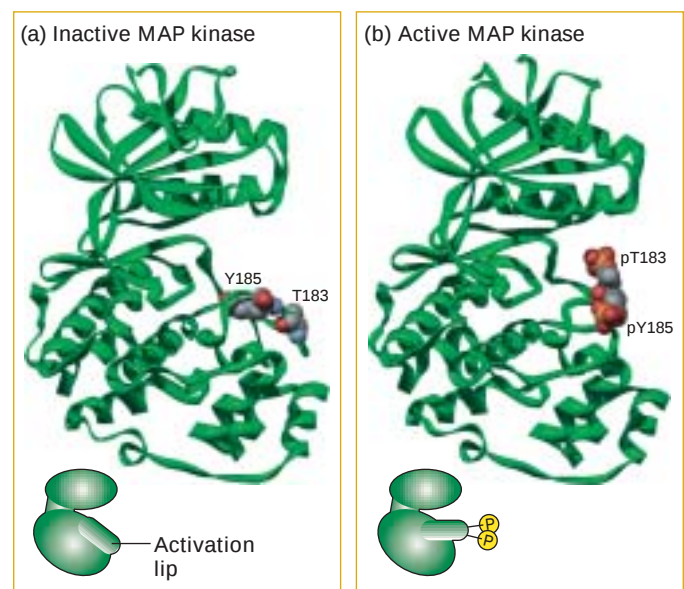
Activation of Raf Kinase The mechanism for activating Raf differs from that used to activate many other protein kinases including MEK and MAP kinase. In a resting cell prior to hormonal stimulation, Raf is present in the cytosol in a conformation in which the N-terminal regulatory domain is bound to the kinase domain, thereby inhibiting its activity. This inactive conformation is stabilized by a dimer of the *14-3-3 protein*, which binds phosphoserine residues in a number of important signaling proteins. Each 14-3-3 monomer binds to a phosphoserine residue in Raf, one to phosphoserine-259 in the N-terminal domain and the other to phosphoserine-621 (see Figure 14-21). These interactions are thought to be essential for Raf to achieve a conformational state such that it can bind to activated Ras.

The binding of Ras·GTP, which is anchored to the membrane, to the N-terminal domain of Raf relieves the inhibition of Raf's kinase activity and also induces a conformational change in Raf that disrupts its association with

14-3-3. Raf phosphoserine-259 then is dephosphorylated (by an unknown phosphatase) and other serine or threonine residues on Raf become phosphorylated by yet other kinases. These reactions incrementally increase the Raf kinase activity by mechanisms that are not fully understood.

Activation of MAP Kinase Biochemical and x-ray crystallographic studies have provided a detailed picture of how phosphorylation activates MAP kinase. As in JAK kinases and the cytosolic domain of receptor tyrosine kinases, the catalytic site in the inactive, unphosphorylated form of MAP kinase is blocked by a stretch of amino acids, the activation lip (Figure 14-22a). Binding of MEK to MAP kinase destabilizes the lip structure, resulting in exposure of tyrosine-185, which is buried in the inactive conformation. Following phosphorylation of this critical tyrosine, MEK phosphorylates the neighboring threonine-183 (Figure 14-22b).

Both the phosphorylated tyrosine and the phosphorylated threonine residues in MAP kinase interact with additional amino acids, thereby conferring an altered conformation to the lip region, which in turn permits binding of ATP to the catalytic site. The phosphotyrosine residue (pY185) also plays a key role in binding specific substrate proteins to the surface of MAP kinase. Phosphorylation promotes not only the catalytic activity of MAP kinase but also



▲ **EXPERIMENTAL FIGURE 14-22 Molecular structures of MAP kinase in its inactive, unphosphorylated form (a) and active, phosphorylated form (b).** Phosphorylation of MAP kinase by MEK at tyrosine-185 (Y185) and threonine-183 (T183) leads to a marked conformational change in the activation lip. This change promotes dimerization of MAP kinase and binding of its substrates, ATP and certain proteins. A similar phosphorylation-dependent mechanism activates JAK kinases, the intrinsic kinase activity of RTKs, and MEK. [After B. J. Canagarajah et al., 1997, *Cell* 90:859.]

its dimerization. The dimeric form of MAP kinase (but not the monomeric form) can be translocated to the nucleus, where it regulates the activity of many nuclear transcription factors.

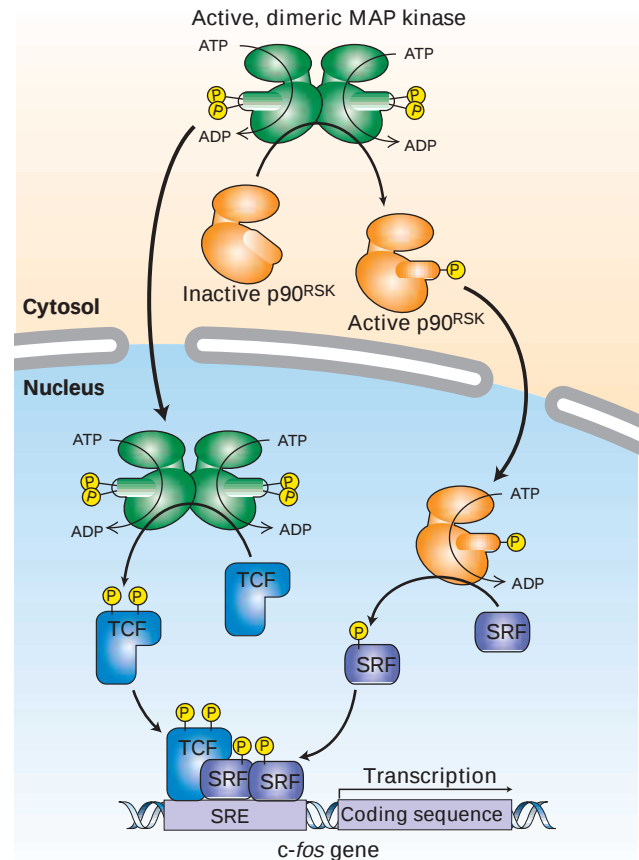
MAP Kinase Regulates the Activity of Many Transcription Factors Controlling Early-Response Genes

Addition of a growth factor (e.g., EGF or PDGF) to quiescent cultured mammalian cells in G_0 causes a rapid increase in the expression of as many as 100 different genes. These are called *early-response genes* because they are induced well before cells enter the S phase and replicate their DNA (see Figure 21-29). One important early-response gene encodes the transcription factor c-Fos. Together with other transcription factors, such as c-Jun, c-Fos induces expression of many genes encoding proteins necessary for cells to progress through the cell cycle. Most RTKs that bind growth factors utilize the MAP kinase pathway to activate genes encoding proteins like c-Fos that propel the cell through the cell cycle.

The enhancer that regulates the *c-fos* gene contains a *serum-response element* (SRE), so named because it is activated by many growth factors in serum. This complex enhancer contains DNA sequences that bind multiple transcription factors. Some of these are activated by MAP kinase, others by different protein kinases that function in other signaling pathways (e.g., protein kinase A in cAMP pathways and protein kinase C in phosphoinositide pathways).

As depicted in Figure 14-23, activated (phosphorylated) dimeric MAP kinase induces transcription of the *c-fos* gene by modifying two transcription factors, *ternary complex factor* (TCF) and *serum response factor* (SRF). In the cytosol, MAP kinase phosphorylates and activates another kinase, $p90^{RSK}$, which translocates to the nucleus, where it phosphorylates a specific serine in SRF. After also translocating to the nucleus, MAP kinase directly phosphorylates specific serines in TCF. Association of phosphorylated TCF with two molecules of phosphorylated SRF forms an active trimeric factor that binds strongly to the SRE DNA segment. As evidence for this model, abundant expression in cultured mammalian cells of a mutant dominant negative TCF that lacks the serine residues phosphorylated by MAP kinase blocks the ability of MAP kinase to activate gene expression driven by the SRE enhancer. Moreover, biochemical studies showed directly that phosphorylation of SRF by active $p90^{RSK}$ increases the rate and affinity of its binding to SRE sequences in DNA, accounting for the increase in the frequency of transcription initiation. Thus both transcription factors are required for maximal growth factor-induced stimulation of gene expression via the MAP kinase pathway, although only TCF is directly activated by MAP kinase.

Phosphorylation of transcription factors by MAP kinase can produce multiple effects on gene expression. For instance, two related *Drosophila* transcription factors, Pointed



▲ **FIGURE 14-23 Induction of gene transcription by activated MAP kinase.** In the cytosol, MAP kinase phosphorylates and activates the kinase $p90^{RSK}$, which then moves into the nucleus and phosphorylates the SRF transcription factor. After translocating into the nucleus, MAP kinase directly phosphorylates the transcription factor TCF. Together, these phosphorylation events stimulate transcription of genes (e.g., *c-fos*) that contain an SRE sequence in their promoter. See the text for details. [See R. Marais et al., 1993, *Cell* **73**:381, and V. M. Rivera et al., 1993, *Mol. Cell Biol.* **13**:6260.]

and Yan, which are directly phosphorylated by MAP kinase, are crucial effectors of RTK signaling in the eye and other tissues. Phosphorylation enhances the activity of Pointed, a transcriptional activator. In contrast, unphosphorylated Yan is a transcriptional repressor that accumulates in the nucleus and inhibits development of R7 cells in the eye. Following signal-induced phosphorylation, Yan accumulates in the cytosol and does not have access to the genes it controls, thereby relieving their repression. Mutant forms of Yan that cannot be phosphorylated by MAP kinase are constitutive repressors of R7 development. This example suggests that a complex interplay among multiple transcription factors, regulated by signal-activated kinases, is critical to cellular development.

G Protein–Coupled Receptors Transmit Signals to MAP Kinase in Yeast Mating Pathways

Although many MAP kinase pathways are initiated by RTKs or cytokine receptors, signaling from other receptors can activate MAP kinase in different cell types of higher eukaryotes. Moreover, yeasts and other single-celled eukaryotes, which lack cytokine receptors or RTKs, do possess several MAP kinase pathways. To illustrate, we consider the mating pathway in *S. cerevisiae*, a well-studied example of a MAP kinase cascade linked to G protein–coupled receptors (GPCRs), in this case for two secreted peptide pheromones, the **a** and α factors.

As discussed in Chapter 22, these pheromones control mating between haploid yeast cells of the opposite mating type, **a** or α . An **a** haploid cell secretes the **a** mating factor and has cell-surface receptors for the α factor; an α cell secretes the α factor and has cell-surface receptors for the **a** factor (see Figure 22-13). Thus each type of cell recognizes the mating factor produced by the opposite type. Activation of the MAP kinase pathway by either the **a** or α receptors in-

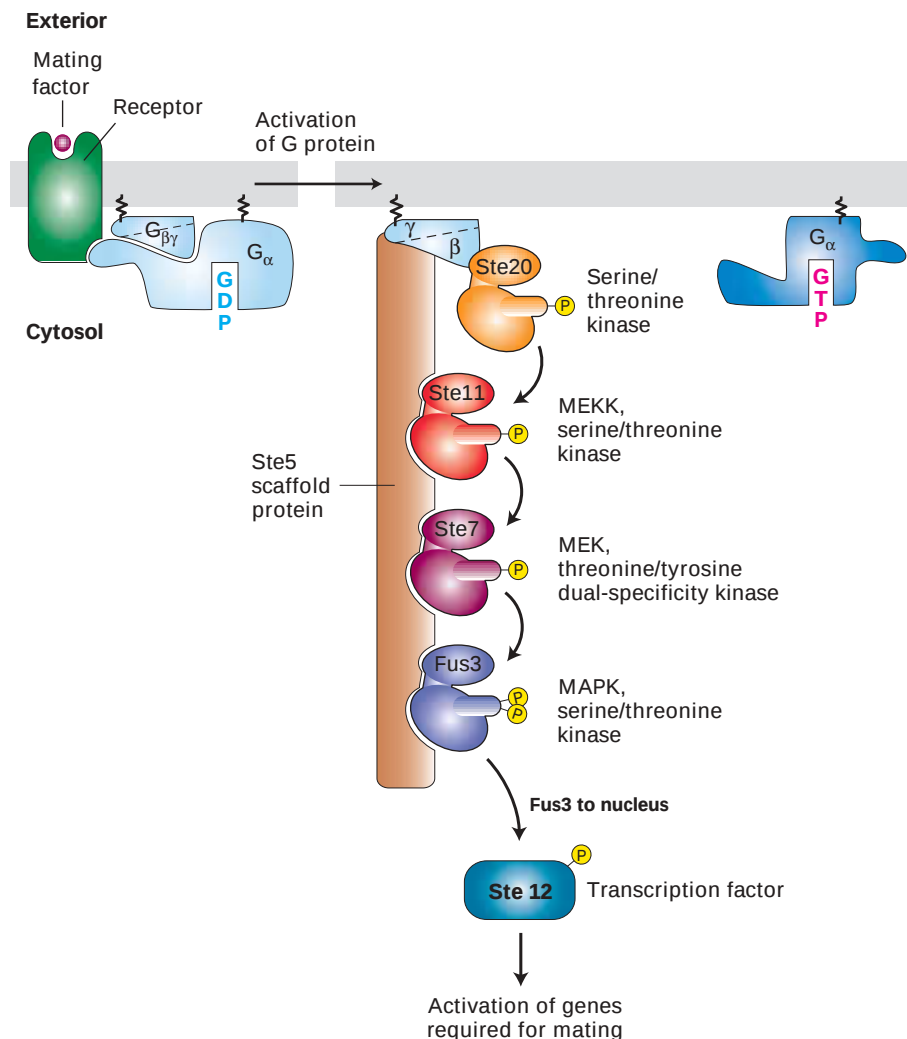
duces transcription of genes that inhibit progression of the cell cycle and others that enable cells of opposite mating type to fuse together and ultimately form a diploid cell.

Ligand binding to either of the two yeast pheromone receptors triggers the exchange of GTP for GDP on the single G_α subunit and dissociation of G_α ·GTP from the $G_{\beta\gamma}$ complex. This activation process is identical to that for the GPCRs discussed in the previous chapter (see Figure 13-11). In most, but not all, mammalian GPCR-initiated pathways, the active G_α transduces the signal. In contrast, mutant studies have shown that the dissociated $G_{\beta\gamma}$ complex mediates all the physiological responses induced by activation of the yeast pheromone receptors. For instance, in yeast cells that lack G_α , the $G_{\beta\gamma}$ subunit is always free. Such cells can mate in the absence of mating factors; that is, the mating response is constitutively on. However, in cells defective for the G_β or G_γ subunit, the mating pathway cannot be induced at all. If dissociated G_α were the transducer, the pathway would be expected to be constitutively active in these mutant cells.

In yeast mating pathways, $G_{\beta\gamma}$ functions by triggering a kinase cascade that is analogous to the one downstream from

► **FIGURE 14-24 Kinase cascade that transmits signals downstream from mating factor receptors in *S. cerevisiae*.**

The receptors for yeast **a** and α mating factors are coupled to the same trimeric G protein. Ligand binding leads to activation and dissociation of the G protein (see Figure 13-10). In the yeast mating pathway, the dissociated $G_{\beta\gamma}$ activates a protein kinase cascade analogous to the cascade downstream of Ras that leads to activation of MAP kinase (see Figure 14-21). The final component, Fus3, is functionally equivalent to MAP kinase (MAPK) in higher eukaryotes. Association of several kinases with the Ste5 scaffold protein contributes to specificity of the signaling pathway by preventing phosphorylation of other substrates. [See A. Whitmarsh and R. Davis, 1998, *Trends Biochem. Sci.* **23**:481, and H. Dohlman and J. Thorner, 2001, *Ann. Rev. Biochem.* **70**:703.]



Ras. The components of this cascade were uncovered mainly through analyses of mutants that possess functional α and β receptors and G proteins but are sterile (*Ste*), or defective in mating responses. The physical interactions between the components were assessed through immunoprecipitation experiments with extracts of yeast cells and other types of studies. Based on these studies, scientists have proposed the kinase cascade depicted in Figure 14-24. $G_{\beta\gamma}$, which is tethered to the membrane via the γ subunit, binds to and activates Ste20, a protein kinase that in turn phosphorylates and activates Ste11, a serine/threonine kinase analogous to Raf and other mammalian MEKK proteins. Activated Ste11 then phosphorylates Ste7, a dual-specificity MEK that then phosphorylates and activates Fus3, a serine/threonine kinase equivalent to MAP kinase. After translocation to the nucleus, Fus3 promotes expression of target genes by phosphorylating and thus activating nuclear transcription factors (e.g., Ste12) that control expression of proteins involved in mating-specific cellular responses. The other component of the yeast mating cascade, Ste5, interacts with $G_{\beta\gamma}$ as well as Ste11, Ste7, and Fus3. Ste5 has no obvious catalytic function and acts as a scaffold for assembling other components in the cascade.

Scaffold Proteins Isolate Multiple MAP Kinase Pathways in Eukaryotic Cells

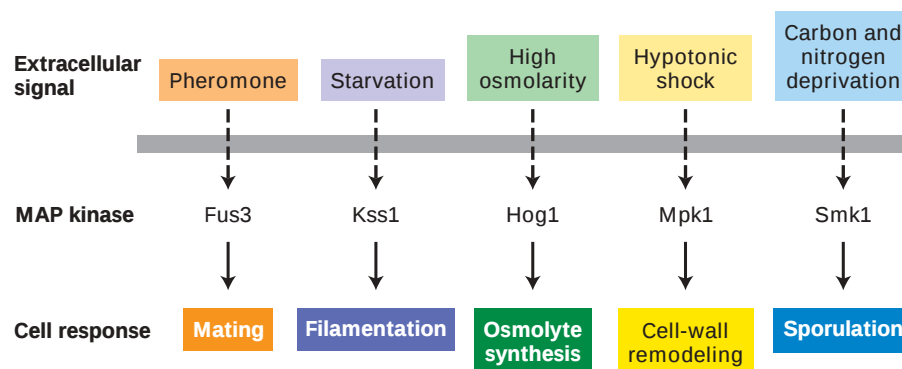
In addition to the MAP kinases discussed above, both yeasts and higher eukaryotic cells contain other members of the MAP kinase superfamily. These include mammalian *Jun N-terminal kinases (JNKs)* and *p38 kinases*, which become activated by various types of stresses, and six yeast kinases described below. Collectively referred to as *MAP kinases*, all these proteins are serine/threonine kinases that are activated in the cytosol in response to specific extracellular signals and then translocate to the nucleus. Activation of all known MAP kinases requires phosphorylation of both a tyrosine and a

threonine residue in the lip region (see Figure 14-22). Similarly, all eukaryotic cells contain several members of the dual-specificity MEK kinase superfamily that phosphorylate different members of the MAP kinase superfamily. Thus in all eukaryotic cells, binding of a wide variety of extracellular signaling molecules triggers highly conserved kinase cascades culminating in activation of a particular MAP kinase. The different MAP kinases mediate specific cellular responses, including morphogenesis, cell death, and stress responses.

Current genetic and biochemical studies in the mouse and *Drosophila* are aimed at determining which MAP kinases are required for mediating the response to which signals in higher eukaryotes. This has already been accomplished in large part for the simpler organism *S. cerevisiae*. Each of the six MAP kinases encoded in the *S. cerevisiae* genome has been assigned by genetic analyses to specific signaling pathways triggered by various extracellular signals, such as pheromones, starvation, high osmolarity, hypotonic shock, and carbon/nitrogen deprivation. Each of these MAP kinases mediates very specific cellular responses (Figure 14-25).

In both yeasts and higher eukaryotic cells, different MAP kinase cascades share some common components. For instance, Ste11 functions in the yeast signaling pathways that regulate mating, filamentous growth, and osmoregulation. Nevertheless, each pathway activates its own MAP kinase: Fus3 in the mating pathway, Kss1 in the filamentation pathway, and Hog1 in the osmoregulation pathway. Similarly, in mammalian cells, common upstream signal-transducing proteins participate in activating multiple JNK kinases.

Once the sharing of components among different MAP kinase pathways was recognized, researchers wondered how the specificity of the cellular responses to particular signals could be achieved. Studies with yeast provided the initial evidence that pathway-specific *scaffold proteins* enable the signal-transducing kinases in a particular pathway to interact with one another but not with kinases in other pathways.



▲ FIGURE 14-25 Overview of five MAP kinase pathways in *S. cerevisiae*. Each pathway is triggered by a specific extracellular signal and leads to activation of a single different MAP kinase, which mediates characteristic cellular responses. Formation of pathway-specific complexes of MAP kinases and

scaffold proteins prevents “cross talk” between pathways containing a common component such as the MEKK Ste11, which occurs in the mating, filamentation, and osmoregulatory pathways (see Figure 14-24). [Adapted from H. D. Madhani and G. R. Fink, 1998, *Trends Genet.* 14(4):152.]

For example, the scaffold protein Ste5 stabilizes a large complex that includes Ste11 and other kinases in the mating pathway (see Figure 14-24). Different Ste11-binding scaffold proteins, however, stabilize signaling complexes containing the components of the filamentation and osmoregulation pathways. In each pathway in which Ste11 participates, it is constrained within a large complex that forms in response to a specific extracellular signal, and signaling downstream from Ste11 is restricted to the complex in which it is localized. As a result, exposure of yeast cells to mating factors induces activation of a single MAP kinase, Fus3, whereas exposure to a high osmolarity or starvation induces activation of different MAP kinases (see Figure 14-25).

Scaffolds for MAP kinase pathways are well documented in yeast, fly, and worm cells, but their presence in mammalian cells has been difficult to demonstrate. Perhaps the best documented scaffold protein is *Ksr* (kinase suppressor of Ras), which binds both MEK and MAP kinase. Loss of the *Drosophila* *Ksr* homolog blocks signaling by a constitutively active Ras protein, suggesting a positive role for *Ksr* in Ras–MAP kinase signaling in fly cells. Although knockout mice that lack *Ksr* are grossly normal, activation of MAP kinase by growth factors or cytokines is lower than normal in several types of cells in these animals. This finding suggests that *Ksr* functions as a scaffold that enhances but is not essential for Ras–MAP kinase signaling in mammalian cells. Other proteins also have been found to bind to specific mammalian MAP kinases. Thus the signal specificity of different MAP kinases in animal cells may arise from their association with various scaffold-like proteins, but much additional research is needed to test this possibility.

KEY CONCEPTS OF SECTION 14.4 MAP Kinase Pathways

- Activated Ras triggers a kinase cascade in which Raf, MEK, and MAP kinase are sequentially phosphorylated and thus activated. Activated MAP kinase dimerizes and translocates to the nucleus (see Figure 14-21).
- Phosphorylation of one or more residues in a conserved lip region activates MAP kinases and many other protein kinases involved in signal-transduction pathways.
- Activation of MAP kinase following stimulation of a growth factor receptor leads to phosphorylation and activation of two transcription factors, TCF and SRF. These associate into a trimeric complex that promotes transcription of various early-response genes (see Figure 14-23).
- Yeast and higher eukaryotes contain multiple MAP kinase pathways that are triggered by activation of various receptor classes including G protein–coupled receptors.
- Different extracellular signals induce activation of different MAP kinases, which regulate diverse cellular processes (see Figure 14-25).

- The upstream components of MAP kinase cascades assemble into large pathway-specific complexes stabilized by scaffold proteins (see Figure 14-24). This assures that activation of one pathway by a particular extracellular signal does not lead to activation of other pathways containing shared components.

14.5 Phosphoinositides as Signal Transducers

In previous sections, we have seen how signal transduction from cytokine receptors and receptor tyrosine kinases (RTKs) begins with formation of multiprotein complexes associated with the plasma membrane. Here we discuss how these receptors initiate signaling pathways that involve membrane-bound phosphorylated inositol lipids, collectively referred to as **phosphoinositides**. We begin with the branch of the phosphoinositide pathway that also is mediated by G protein–coupled receptors and then consider another branch that is not shared with these receptors.

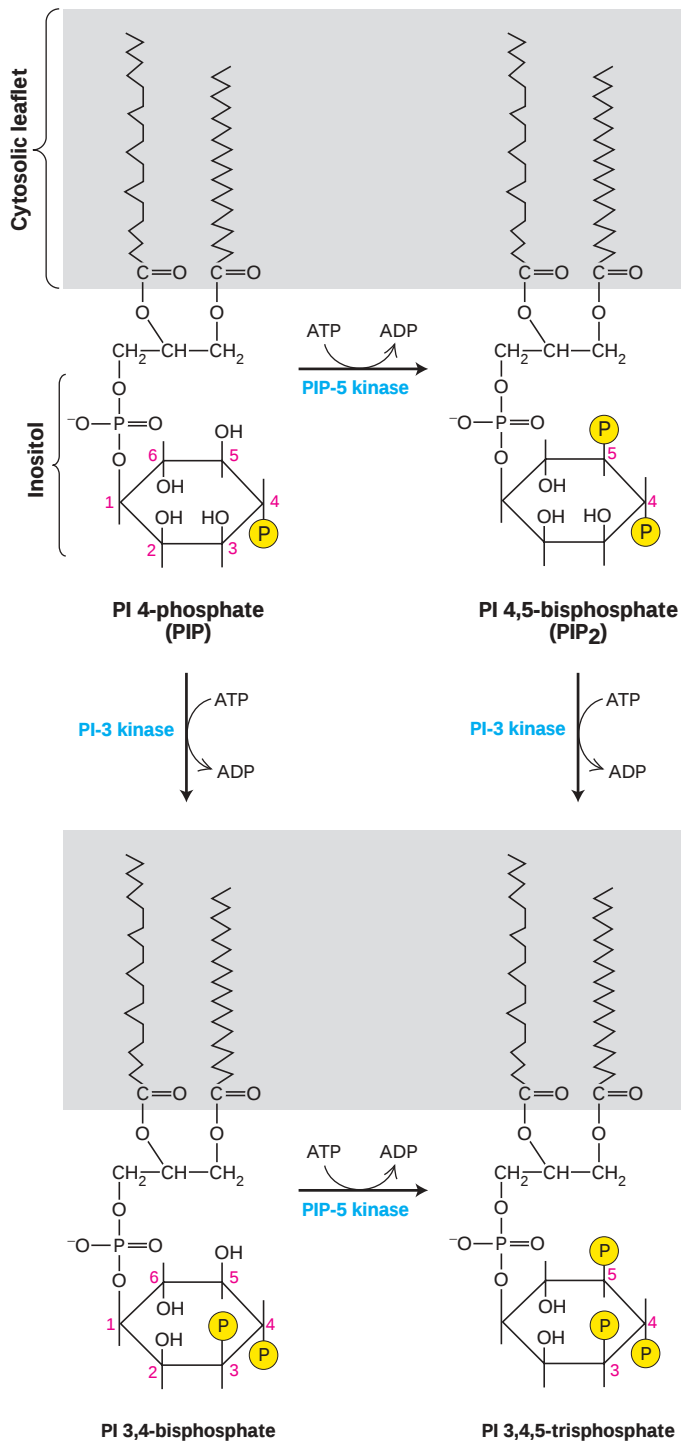
Phospholipase C_γ Is Activated by Some RTKs and Cytokine Receptors

As discussed in Chapter 13, hormonal stimulation of some G protein–coupled receptors leads to activation of the β isoform of phospholipase C (PLC _{β}). This membrane-associated enzyme then cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate two important **second messengers**, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). Signaling via the *IP₃/DAG pathway* leads to an increase in cytosolic Ca²⁺ and to activation of **protein kinase C** (see Figure 13-29).

Many RTKs and cytokine receptors also can initiate the IP₃/DAG pathway by activating another isoform of phospholipase C, the γ isoform (PLC _{γ}). The SH2 domains of PLC _{γ} bind to specific phosphotyrosines of the activated receptors, thus positioning the enzyme close to its membrane-bound substrate PIP₂ (see Figure 13-28). In addition, the receptor kinase activity phosphorylates tyrosine residues on the bound PLC _{γ} , enhancing its hydrolase activity. Thus activated RTKs and cytokine receptors promote PLC _{γ} activity in two ways: by localizing the enzyme to the membrane and by phosphorylating it.

Recruitment of PI-3 Kinase to Hormone-Stimulated Receptors Leads to Activation of Protein Kinase B

In addition to initiating the IP₃/DAG pathway, some activated RTKs and cytokine receptors can initiate another phosphoinositide pathway, the *PI-3 kinase pathway*, by re-



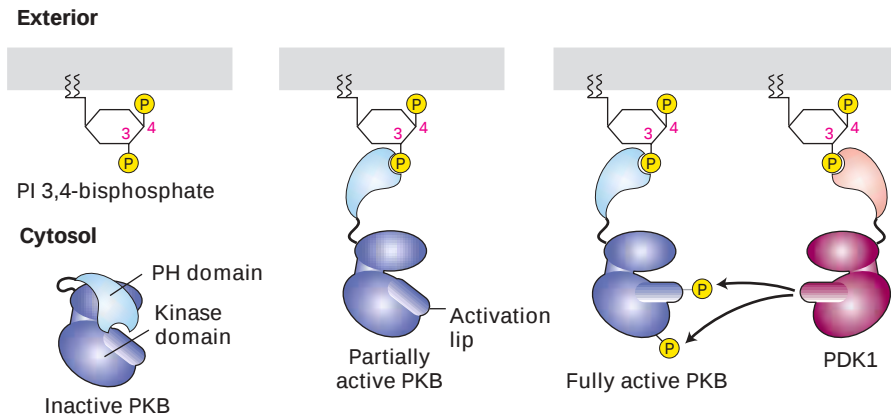
▲ **FIGURE 14-26 Generation of phosphatidylinositol 3-phosphates.** The enzyme phosphatidylinositol-3 kinase (PI-3 kinase) is recruited to the membrane by many activated receptor tyrosine kinases (RTKs) and cytokine receptors. The 3-phosphate added by this enzyme is a binding site for various signal-transduction proteins. [See L. Rameh and L. C. Cantley, 1999, *J. Biol. Chem.* **274**:8347.]

cruiting the enzyme phosphatidylinositol-3 kinase to the membrane. PI-3 kinase was first identified as a kinase that copurifies with several viral oncoproteins such as the “middle T” protein encoded by polyoma virus. When inactive, dominant negative, versions of PI-3 kinase are expressed in virus-transformed cells, they inhibit the uncontrolled cell proliferation characteristic of virus-transformed cells. This finding suggested that the normal kinase is important in certain signaling pathways essential for cell proliferation or for the prevention of apoptosis. Subsequent work showed that PI-3 kinases participate in many signaling pathways related to cell growth and apoptosis. Of the nine PI-3 kinase homologs encoded by the human genome, the best characterized contains a p110 subunit with catalytic activity and a p85 subunit with an SH2 domain.

The SH2 domain in PI-3 kinase binds to phosphotyrosine residues in the cytosolic domain of many activated RTKs and cytokine receptors. The recruitment of PI-3 kinase to the plasma membrane by activated receptors positions its catalytic domain near its phosphoinositide substrates on the cytosolic face of the plasma membrane, leading to formation of PI 3,4-bisphosphate or PI 3,4,5-trisphosphate (Figure 14-26). By acting as docking sites for various signal-transducing proteins, these membrane-bound PI 3-phosphates in turn transduce signals downstream in several important pathways.

A primary binding target of PI 3-phosphates is **protein kinase B (PKB)**, a serine/threonine kinase. Besides its kinase domain, PKB contains a PH domain that tightly binds the 3-phosphate in both PI 3,4-bisphosphate and PI 3,4,5-trisphosphate. In unstimulated, resting cells, the level of both these compounds is low, and protein kinase B is present in the cytosol in an inactive form. Following hormone stimulation and the resulting rise in PI 3-phosphates, protein kinase B binds to them and is localized at the cell surface membrane.

Binding of protein kinase B to PI 3-phosphates not only recruits the enzyme to the plasma membrane but also releases inhibition of the catalytic site by the PH domain in the cytosol. Maximal activation of protein kinase B, however, depends on recruitment of another kinase, PDK1, to the plasma membrane via binding of its PH domain to PI 3-phosphates. Both membrane-associated protein kinase B and PDK1 can diffuse in the plane of the membrane, bringing them close enough so that PDK1 can phosphorylate protein kinase B (Figure 14-27). PDK1 phosphorylates one serine residue in the activation lip of protein kinase B, providing yet another example of kinase activation by phosphorylation in this segment. Phosphorylation of a second serine, not in the lip segment, is necessary for maximal protein kinase B activity. Thus, as with Raf, an inhibitory domain and phosphorylation by other kinases regulate the activity of protein kinase B. Once fully activated, protein kinase B can dissociate from the plasma membrane and phosphorylate its many target proteins.



▲ **FIGURE 14-27 Recruitment and activation of protein kinase B (PKB) in PI-3 kinase pathways.** In unstimulated cells, PKB is in the cytosol with its PH domain bound to the catalytic domain, inhibiting its activity. Hormone stimulation leads to activation of PI-3 kinase and subsequent formation of phosphatidylinositol

(PI) 3-phosphates (see Figure 14-26). The 3-phosphate groups serve as docking sites on the plasma membrane for the PH domain of PKB and another kinase, PDK1. Full activation of PKB requires phosphorylation both in the activation lip and at the C-terminus by PDK1. [Adapted from A. Tokar and A. Newton, 2000, *Cell* **103**:185, and M. Scheid et al., 2002, *Mol. Cell Biol.* **22**:6247.]

The Insulin Receptor Acts Through the PI-3 Kinase Pathway to Lower Blood Glucose

The insulin receptor is a dimeric receptor tyrosine kinase that can initiate the Ras–MAP kinase pathway, leading to changes in gene expression. Insulin stimulation also can initiate the PI-3 kinase pathway just described, leading to activation of protein kinase B. In insulin-stimulated liver, muscle, and fat cells, activated protein kinase B acts in several ways to lower blood glucose and promote glycogen synthesis.

The principal mechanism by which insulin causes a reduction of the blood glucose level is by increasing import of glucose by fat and muscle cells. This effect is mediated by protein kinase B, which through mechanisms that are not fully understood causes movement of the GLUT4 glucose transporter from intracellular membranes to the cell surface (Chapter 15). The resulting increased influx of glucose into these cells lowers blood glucose levels.

In both liver and muscle, insulin stimulation also leads to activation of glycogen synthase (GS), which synthesizes glycogen from UDP-glucose (see Figure 13-16). This represents another mechanism for reducing glucose concentration in the circulation. In resting cells (i.e., in the absence of insulin), glycogen synthase kinase 3 (GSK3) is active and phosphorylates glycogen synthase, thereby blocking its activity. Activated protein kinase B phosphorylates and thereby inactivates GSK3. As a result, GSK3-mediated inhibition of glycogen synthase is relieved, promoting glycogen synthesis.

Activated Protein Kinase B Promotes Cell Survival by Several Pathways

In many cells activated protein kinase B directly phosphorylates pro-apoptotic proteins such as Bad, thereby preventing

activation of an apoptotic pathway leading to cell death (Chapter 22). Activated protein kinase B also promotes survival of many cultured cells by phosphorylating the transcription factor Forkhead-1 on as many as three serine or threonine residues. In the absence of growth factors, Forkhead-1 is unphosphorylated and localizes to the nucleus, where it activates transcription of several genes encoding pro-apoptotic proteins. When growth factors are added to the cells, protein kinase B becomes active and phosphorylates Forkhead-1. This allows the cytosolic phosphoserine-binding protein 14-3-3 to bind Forkhead-1 and thus sequester it in the cytosol. (14-3-3 is the same protein that retains phosphorylated Raf protein in the cytosol; see Figure 14-21.) Withdrawal of growth factor leads to inactivation of protein kinase B and dephosphorylation of Forkhead-1, thus favoring apoptosis. A Forkhead-1 mutant in which the three serine target residues for protein kinase B are mutated is “constitutively active” and initiates apoptosis even in the presence of activated protein kinase B. This finding demonstrates the importance of Forkhead-1 in controlling apoptosis of cultured cells.

PTEN Phosphatase Terminates Signaling via the PI-3 Kinase Pathway

Like virtually all intracellular signaling events, phosphorylation by PI-3 kinase is reversible. The relevant phosphatase, termed *PTEN phosphatase*, has an unusually broad specificity. Although PTEN can remove phosphate groups attached to serine, threonine, and tyrosine residues in proteins, its ability to remove the 3-phosphate from PI 3,4,5-trisphosphate is thought to be its major function in cells. Overexpression of PTEN in cultured mammalian cells promotes apoptosis by reducing the level of PI 3,4,5-trisphosphate and hence the activation and anti-apoptotic effect of protein kinase B.



The gene encoding PTEN is deleted in multiple types of advanced human cancers, and its loss is thought to lead to uncontrolled growth. Indeed, cells lacking PTEN have elevated levels of PI 3,4,5-trisphosphate and PKB activity. Since protein kinase B exerts an anti-apoptotic effect, loss of PTEN indirectly reduces the programmed cell death that is the normal fate of abnormally controlled cells. In certain cells, such as neuronal stem cells, absence of PTEN not only prevents apoptosis but also leads to stimulation of cell-cycle progression and an enhanced rate of proliferation. Thus knockout mice that cannot express PTEN have big brains with excess numbers of neurons, attesting to PTEN's importance in control of normal development. ■

The Receptor for a Particular Growth Factor Often Is Linked to Multiple Signaling Pathways

Interaction of different signaling pathways permits the fine-tuning of cellular activities required to carry out complex developmental and physiological processes. As we have noted previously, both RTKs and cytokine receptors can initiate signaling via the Ras–MAP kinase pathway, DAG/IP₃ pathway, and PI-3 kinase pathway (see Table 14-1). In addition, cytokine receptors can act through their associated JAK kinases to directly activate STAT transcription factors.

Activation of multiple signal-transduction pathways by many receptors allows different sets of genes to be independently controlled by the same or different receptors. Occasionally these pathways can induce opposite effects. For example, genetic manipulation of the Ras–MAP kinase and PI-3 kinase pathways during muscle differentiation indicates that these pathways have opposite phenotypic effects: activation of the Ras–MAP kinase pathway inhibits myocyte differentiation into myotubes, whereas activation of the PI-3 kinase pathway promotes it.

The initiation of tissue-specific signaling pathways by stimulation of the *same* receptor in different cells is exemplified by the EGF receptor. Genetic studies analogous to those described earlier for development of R7 cells in *Drosophila* demonstrated the central importance of EGF-stimulated signaling via the Ras–MAP pathway in development of the vulva in *C. elegans*. Other genetic studies, however, showed that stimulation of the EGF receptor triggers a Ras-independent pathway in some tissues. For example, one of the many functions of EGF in *C. elegans* is to control contractility of smooth muscle, which in turn regulates the extrusion of oocytes from one compartment of the hermaphrodite gonad to another, where they are fertilized. Coupling of the EGF receptor to Ras is not required for the EGF-induced contractions of the gonad. Analysis of several different types of mutations led researchers to conclude that in *C. elegans* smooth muscle, the EGF receptor is linked to the IP₃/DAG pathway. Ligand binding to the receptor leads to activation of PLC γ activity, an increase in IP₃, and release of intracellular Ca²⁺ stores. The increased cytosolic Ca²⁺ level then promotes muscle contraction.

In Chapter 15 we will encounter several other examples of how stimulation of the same receptor in different cell types activates different signaling pathways that produce very diverse effects on the metabolism and fate of the cell.

KEY CONCEPTS OF SECTION 14.5

Phosphoinositides as Signal Transducers

- Many RTKs and cytokine receptors can initiate the IP₃/DAG signaling pathway by activating phospholipase C γ (PLC γ), a different PLC isoform than the one activated by G protein–coupled receptors.
- Activated RTKs and cytokine receptors can initiate another phosphoinositide pathway by binding PI-3 kinases, thereby allowing the catalytic subunit access to its membrane-bound phosphoinositide (PI) substrates, which are phosphorylated at the 3 position (see Figure 14-26).
- The PH domain in various proteins binds to PI 3-phosphates, forming signaling complexes associated with the plasma membrane.
- Protein kinase B (PKB) becomes partially activated by binding to PI 3-phosphates. Its full activation requires phosphorylation by another kinase (PDK1), which also is recruited to the membrane by binding to PI 3-phosphates (see Figure 14-27).
- Activated protein kinase B promotes survival of many cells by directly inactivating several pro-apoptotic proteins and down-regulating expression of others.
- Signaling via the PI-3 kinase pathway is terminated by the PTEN phosphatase, which hydrolyzes the 3-phosphate in PI 3-phosphates. Loss of PTEN, a common occurrence in human tumors, promotes cell survival and proliferation.
- A single RTK or cytokine receptor often initiates different signaling pathways in multiple cell types. Different pathways may be essential in certain cell signaling events but not in others.

14.6 Pathways That Involve Signal-Induced Protein Cleavage

Up to now we have discussed reversible signaling pathways, where inactivation is as important as the initial activation. In contrast are essentially irreversible pathways in which a component is proteolytically cleaved. Here we consider two such pathways: the *NF- κ B* pathway, which enables cells to respond immediately and vigorously to a number of stress-inducing conditions, and the *Notch/Delta* pathway, which determines the fates of many types of cells during development. Proteolytic activation of the cell-surface receptor Notch is facilitated by presenilin 1, a membrane protein that also has been implicated in the pathology of Alzheimer's disease.

Signal-Induced Degradation of a Cytosolic Inhibitor Protein Activates the NF- κ B Transcription Factor

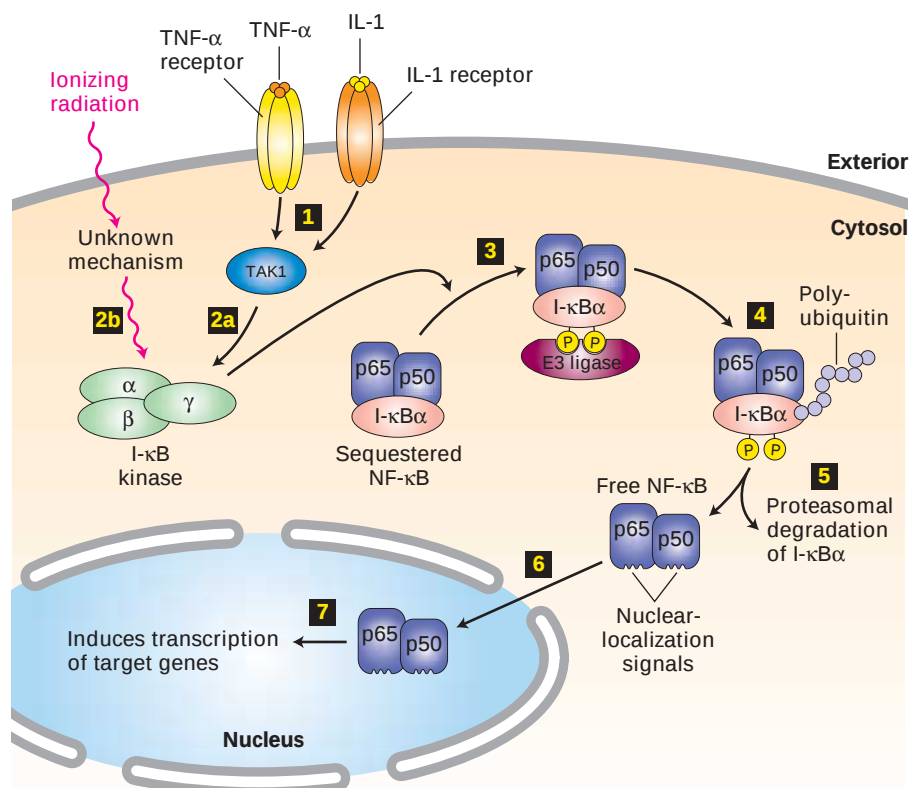
The examples in previous sections have demonstrated the importance of signal-induced phosphorylation in modulating the activity of many transcription factors. Another mechanism for regulating transcription factor activity in response to extracellular signals was revealed in studies with both mammalian cells and *Drosophila*. This mechanism, which involves phosphorylation and subsequent ubiquitin-mediated degradation of an inhibitor protein, is exemplified by the NF- κ B transcription factor.

Originally discovered on the basis of its transcriptional activation of the gene encoding the κ light-chain of antibodies (immunoglobulins) in B cells, NF- κ B is now thought to be the master transcriptional regulator of the immune system in mammals. Although flies do not make antibodies, NF- κ B homologs in *Drosophila* mediate the immune response to bacterial and viral infection by inducing synthesis of a large number of antimicrobial peptides that are secreted from cells. This indicates that the NF- κ B regulatory system is more than half a billion years old. NF- κ B is rapidly activated in mammalian immune-system cells in response to infection, inflammation, and a number of other stressful situations, such as ionizing radiation. It also is activated by so-called inflammatory cytokines such as tumor necrosis factor α (TNF- α) and interleukin 1 (IL-1), which are released by nearby cells in response to infection.

Biochemical studies in mammalian cells and genetic studies in flies have provided important insights into the operation of the NF- κ B pathway (Figure 14-28). The two subunits of heterodimeric NF- κ B (p65 and p50) share a region of homology at their N-termini that is required for their dimerization and binding to DNA. In resting cells, NF- κ B is sequestered in an inactive state in the cytosol by direct binding to an inhibitor called I- κ B. A single molecule of I- κ B binds to the N-terminal domains of each subunit in the p50/p65 heterodimer, thereby masking the nuclear-localization signals. A protein kinase complex termed *I- κ B kinase* is the point of convergence of all of the extracellular signals that activate NF- κ B. Within minutes of stimulation, I- κ B kinase becomes activated and phosphorylates two N-terminal serine residues on I- κ B. An E3 ubiquitin ligase then binds to these phosphoserines and polyubiquitinates I- κ B, triggering its immediate degradation by a proteasome (see Figure 3-13). In cells expressing mutant forms of I- κ B in which these two serines have been changed to alanine, and thus cannot be phosphorylated, NF- κ B is permanently repressed, demonstrating that phosphorylation of I- κ B is essential for pathway activation.

The degradation of I- κ B exposes the nuclear-localization signals on NF- κ B, which then translocates into the nucleus and activates transcription of a multitude of target genes. Despite its activation by proteolysis, NF- κ B signaling eventually is turned off by a negative feedback loop, since one of the genes whose transcription is immediately induced by NF- κ B encodes I- κ B. The resulting increased levels of the

► **FIGURE 14-28 NF- κ B signaling pathway.** In resting cells, the dimeric transcription factor NF- κ B, composed of p50 and p65, is sequestered in the cytosol, bound to the inhibitor I- κ B. Stimulation by TNF- α or IL-1 induces activation of TAK1 kinase (step 1), leading to activation of the trimeric I- κ B kinase (step 2a). Ionizing radiation and other stresses can directly activate I- κ B kinase by an unknown mechanism (step 2b). Following phosphorylation of I- κ B by I- κ B kinase (step 3), polyubiquitination of I- κ B (step 4) targets it for degradation by proteasomes (step 5). The removal of I- κ B unmasks the nuclear-localization signals (NLS) in both subunits of NF- κ B, allowing their translocation to the nucleus (step 6). Here NF- κ B activates transcription of numerous target genes (step 7), including the gene encoding the α subunit of I- κ B, which acts to terminate signaling. [See M. Karin and Y. Ben-Neriah, 2000, *Ann. Rev. Immunol.* **18**:621, and R. Khush, F. Leulier, and B. Lemaitre, 2001, *Trends Immunol.* **22**:260.]



I- κ B protein bind active NF- κ B in the nucleus and return it to the cytosol.

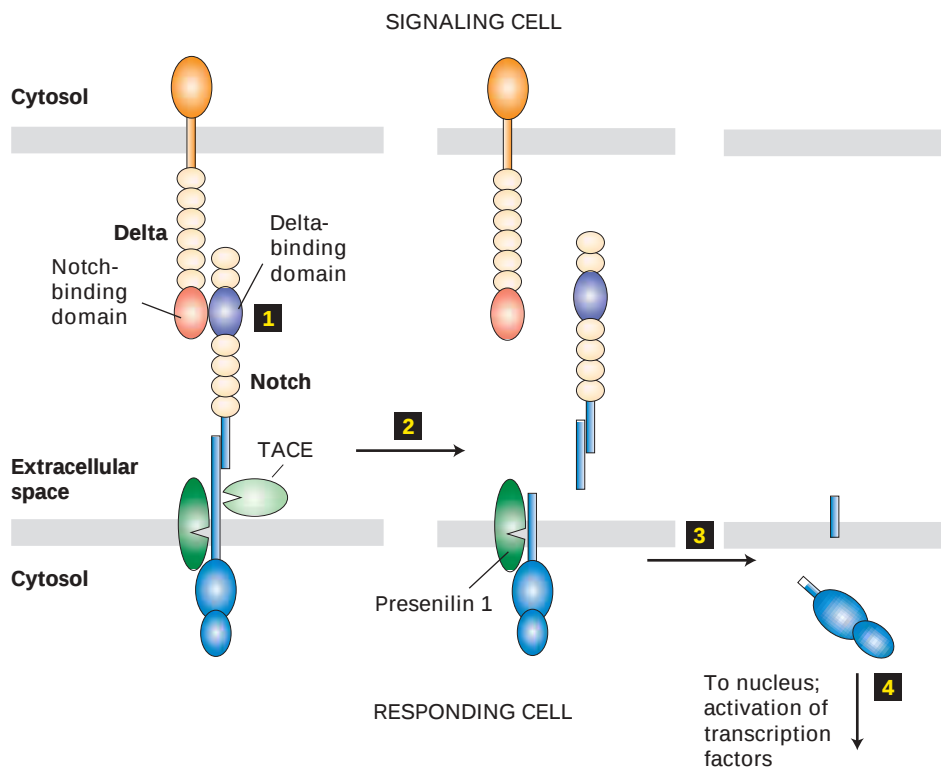
NF- κ B stimulates transcription of more than 150 genes, including those encoding cytokines and chemokines that attract other immune-system cells and fibroblasts to sites of infection. It also promotes expression of receptor proteins that enable neutrophils (a type of white blood cell) to migrate from the blood into the underlying tissue (see Figure 6-30). In addition, NF- κ B stimulates expression of iNOS, the inducible isoform of the enzyme that produces nitric oxide, which is toxic to bacterial cells, and of several anti-apoptotic proteins, which prevent cell death. Thus this single transcription factor coordinates and activates the body's defense either directly by responding to pathogens and stress or indirectly by responding to signaling molecules released from other infected or wounded tissues and cells.

Besides its roles in inflammation and immunity, NF- κ B plays a key role during mammalian development. For instance, mouse embryos that cannot express one of the I- κ B kinase subunits die at mid-gestation of liver degeneration caused by excessive apoptosis of cells that would normally survive; thus NF- κ B is essential for normal development of this tissue. As we will see in Chapter 21, phosphorylation-dependent degradation of a cyclin kinase-dependent inhibitor plays a central role in regulating progression through the cell cycle in *S. cerevisiae*. It seems likely that phosphorylation-dependent protein degradation may emerge as a common regulatory mechanism in many different cellular processes.

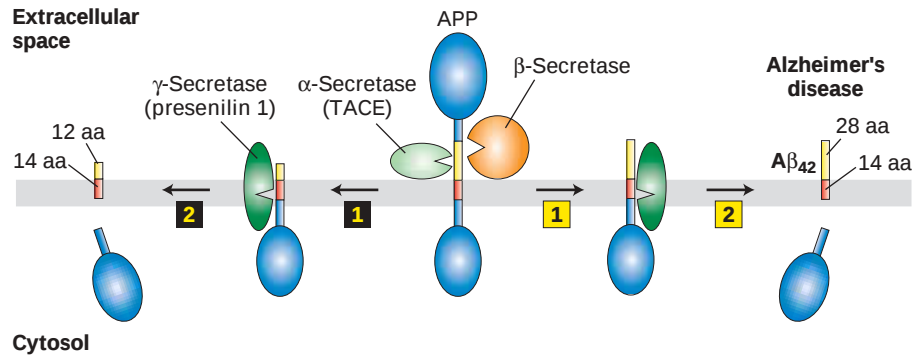
Regulated Intramembrane Proteolysis Catalyzed by Presenilin 1 Activates Notch Receptor

Both Notch and its ligand Delta are transmembrane proteins with numerous EGF-like repeats in their extracellular domains. They participate in a highly conserved and important type of cell differentiation in both invertebrates and vertebrates, called **lateral inhibition**, in which adjacent and developmentally equivalent cells assume completely different fates. This process, discussed in detail in Chapter 15, is particularly important in preventing too many nerve precursor cells forming from an undifferentiated layer of epithelial cells.

Notch protein is synthesized as a monomeric membrane protein in the endoplasmic reticulum, where it binds *presenilin 1*, a multispanning membrane protein; the complex travels first to the Golgi and then on to the plasma membrane. In the Golgi, Notch undergoes a proteolytic cleavage that generates an extracellular subunit and a transmembrane-cytosolic subunit; the two subunits remain noncovalently associated with each other in the absence of interaction with Delta residing on another cell. Binding of Notch to Delta triggers two proteolytic cleavages in the responding cell (Figure 14-29). The second cleavage, within the hydrophobic membrane-spanning region of Notch, is catalyzed by presenilin 1 and releases the Notch cytosolic segment, which immediately translocates to the nucleus. Such signal-induced *regulated intramembrane proteolysis (RIP)* also occurs in the response of cells to high cholesterol (Chapter 18) and to the presence of unfolded proteins in the endoplasmic reticulum (Chapter 16).



◀ **FIGURE 14-29 Notch/Delta signaling pathway.** The extracellular subunit of Notch on the responding cell is noncovalently associated with its transmembrane-cytosolic subunit. Binding of Notch to its ligand Delta on an adjacent signaling cell (step 1) first triggers cleavage of Notch by the membrane-bound metalloprotease TACE (tumor necrosis factor alpha converting enzyme), releasing the extracellular segment (step 2). Presenilin 1, an integral membrane protein, then catalyzes an intramembrane cleavage that releases the cytosolic segment of Notch (step 3). Following translocation to the nucleus, this Notch segment interacts with several transcription factors that act to affect expression of genes that in turn influence the determination of cell fate during development (step 4). [See M. S. Brown et al., 2000, *Cell* 100:391, and Y.-M. Chan and Y. Jan, 1999, *Neuron* 23:201.]



▲ **FIGURE 14-30 Proteolytic cleavage of APP, a neuronal plasma membrane protein.** (Left) Sequential proteolytic cleavage by α -secretase (step 1) and γ -secretase (step 2) produces an innocuous membrane-embedded peptide of 26 amino acids. γ -Secretase is a complex of several proteins, but the proteolytic site that catalyzes intramembrane cleavage probably resides within presenilin 1. (Right) Cleavage in the

extracellular domain by β -secretase (step 1) followed by cleavage within the membrane by γ -secretase generates the 42-residue $A\beta_{42}$ peptide that has been implicated in formation of amyloid plaques in Alzheimer's disease. In both pathways the cytosolic segment of APP is released into the cytosol, but its function is not known. [See W. Esler and M. Wolfe, 2001, *Science* 293:1449, and C. Haass and H. Steiner, 2002, *Trends Cell Biol.* 12:556.]

In *Drosophila* the released intracellular segment of Notch forms a complex with a DNA-binding protein called Suppressor of Hairless, or Su(H), and stimulates transcription of many genes whose net effect is to influence the determination of cell fate during development. One of the proteins increased in this manner is Notch itself, and Delta production is correspondingly reduced (see Figure 15-38). As we see in Chapter 15, reciprocal regulation of the receptor and ligand in this fashion is an essential feature of the interaction between initially equivalent cells that causes them to assume different cell fates.



Presenilin 1 (PS1) was first identified as the product of a gene that commonly is mutated in patients with an early-onset autosomal dominant form of Alzheimer's disease. A major pathologic change associated with Alzheimer's disease is accumulation in the brain of *amyloid plaques* containing aggregates of a small peptide containing 42 residues termed $A\beta_{42}$. This peptide is derived by proteolytic cleavage of APP (amyloid precursor protein), a cell-surface protein of unknown function expressed by neurons. APP actually undergoes cleavage by two pathways (Figure 14-30). In each pathway the initial cleavage occurs within the extracellular domain, catalyzed by α - or β -secretase; γ -secretase then catalyzes a second cleavage at the same intramembrane site in both pathways. The pathway initiated by α -secretase, which involves the same membrane-bound metalloprotease TACE that cleaves Notch, generates a 26-residue peptide that apparently does no harm. The pathway initiated by β -secretase generates the pathologic $A\beta_{42}$. The missense mutations in presenilin 1 involved in Alzheimer's disease enhance the formation of the $A\beta_{42}$ peptide, leading to plaque formation and eventually to the death of neurons.

Evidence supporting the involvement of presenilin 1 in Notch signaling (see Figure 14-29) came from genetic studies in the roundworm *C. elegans*. Mutations in the worm homolog of presenilin 1 caused developmental defects similar to those caused by Notch mutations. Later work showed that mammalian Notch does not undergo signal-induced intramembrane proteolysis in mouse neuronal cells genetically missing presenilin 1. But whether presenilin 1 is the actual γ -secretase protease or an essential cofactor of the "real" protease is not yet certain, since presenilin 1 is part of a large complex containing several other integral membrane proteins. Within its membrane-spanning segments, presenilin 1 has two aspartate residues in a configuration that resembles that of the two aspartates in the active site of water-soluble "aspartyl proteases," and mutation of either of these aspartate residues in presenilin 1 abolishes its ability to stimulate cleavage of Notch. Similarly, a battery of chemical protease inhibitors blocks cleavage of Notch and γ -secretase cleavage of APP with the same potency, suggesting that the same protease is involved. Current data are thus consistent with the notion that presenilin 1 is the protease that cleaves both Notch and APP within their transmembrane segments. However, cleavage of both Notch and APP occurs at or near the plasma membrane, whereas the majority of presenilin is found in the endoplasmic reticulum. This finding suggests that presenilin may act in conjunction with other proteins in the unusual intramembrane proteolysis of Notch and APP. ■

KEY CONCEPTS OF SECTION 14.6

Pathways That Involve Signal-Induced Protein Cleavage

- The NF- κ B transcription factor regulates many genes that permit cells to respond to infection and inflammation.

- In unstimulated cells, NF- κ B is localized to the cytosol, bound to an inhibitor protein, I- κ B. In response to extracellular signals, phosphorylation-dependent ubiquitination and degradation of I- κ B in proteasomes releases active NF- κ B, which translocates to the nucleus (see Figure 14-28).
- Upon binding to its ligand Delta on the surface of an adjacent cell, the Notch receptor protein undergoes two proteolytic cleavages. The released Notch cytosolic segment then translocates into the nucleus and modulates gene transcription (see Figure 14-29).
- Presenilin 1, which catalyzes the regulated intramembrane cleavage of Notch, also participates in the cleavage of amyloid precursor protein (APP) into a peptide that forms plaques characteristic of Alzheimer's disease.

14.7 Down-Modulation of Receptor Signaling

We have already seen several ways that signal-transduction pathways can be regulated. The levels of hormones produced and released from signaling cells are adjusted constantly to meet the needs of the organism. For example, kidney cells make and secrete more erythropoietin when the oxygen level is low and more red blood cells are needed. Intracellular proteins such as Ski and SOCS are induced following stimulation by TGF β or cytokines, and then negatively regulate their respective signal-transduction pathways. Phosphorylation of receptors and downstream signaling proteins are reversed by the carefully controlled action of phosphatases. Here we discuss two other mechanisms by which signaling pathways are down-regulated: removal of receptors from the cell surface by endocytosis, and secretion of proteins that bind and sequester hormones, thus preventing their interaction with cell-surface receptors.

Endocytosis of Cell-Surface Receptors Desensitizes Cells to Many Hormones

In previous sections we discussed several signal-transduction pathways activated immediately after stimulation of cytokine receptors and receptor tyrosine kinases (RTKs). If the level of hormone in the environment remains high for several hours, cells usually undergo *desensitization*, such that they no longer respond to that concentration of hormone. This prevents inappropriate prolonged receptor activity, but under these conditions cells usually will respond if the hormone level is increased further. Ligand-dependent **receptor-mediated endocytosis**, which reduces the number of available cell-surface receptors, is a principal way that cells are desensitized to many peptides and other hormones.

In the absence of EGF ligand, for instance, the EGF receptor is internalized at a relatively slow rate by bulk mem-

brane flow. Besides activating the receptor's protein tyrosine kinase, binding to EGF induces a conformational change in the cytosolic tail of the receptor. This exposes a sorting motif that facilitates receptor recruitment into clathrin-coated pits and subsequent internalization. After internalization, some cell-surface receptors (e.g., the LDL receptor) are efficiently recycled to the surface (see Figure 17-28). In contrast, internalized receptors for many peptide hormones, together with their bound hormone ligands, commonly are transported to lysosomes wherein they are degraded, rather than being recycled to the cell surface.

For example, each time an EGF receptor is internalized with bound EGF, it has about a 50 percent chance of being degraded. Exposure of a fibroblast cell to high levels of EGF for 1 hour induces several rounds of endocytosis, resulting in degradation of most receptor molecules. If the concentration of extracellular EGF is then reduced, the number of EGF receptors on the cell surface recovers by synthesis of new receptor molecules, a slow process that may take more than a day. In this way a cell can become desensitized to a continual high level of hormone and, after hormone removal, reestablish its initial level of cell-surface receptors, thereby becoming sensitive again to a low level of hormone.

Experiments with mutant cell lines demonstrate that internalization of RTKs plays an important role in regulating cellular responses to EGF and other growth factors. For instance, a mutation in the EGF receptor that prevents it from being incorporated into coated pits, and thus makes it resistant to ligand-induced endocytosis, substantially increases the sensitivity of cells to EGF as a mitogenic signal. Such mutant cells are prone to EGF-induced cell transformation. Interestingly, internalized receptors can continue to signal from intracellular compartments prior to their degradation.



In most cases, peptide hormones that are internalized bound to their receptors are degraded intracellularly. If the initial extracellular hormone level is relatively low, this process may reduce the hormone level sufficiently to terminate cell signaling after a few hours or so. For instance, IL-2, a cytokine that stimulates growth of immune T cells, normally is depleted from the extracellular environment by this mechanism, leading to cessation of signaling. Mutant forms of IL-2 have been obtained that bind to the IL-2 receptor normally at pH 7.5, that of the extracellular medium, but poorly at pH 6, that of the initial endocytic vesicle, or endosome. These mutant IL-2 proteins dissociate from the receptor in the endosome and are “recycled”; that is, they are secreted back into the extracellular medium rather than accompanying the receptor to the lysosome for degradation. Because the lifetime of these mutant IL-2 proteins is longer than normal, they are more potent than their normal counterparts and may be useful therapeutically for stimulating production of T cells. ■

Secreted Decoy Receptors Bind Hormone and Prevent Receptor Activation

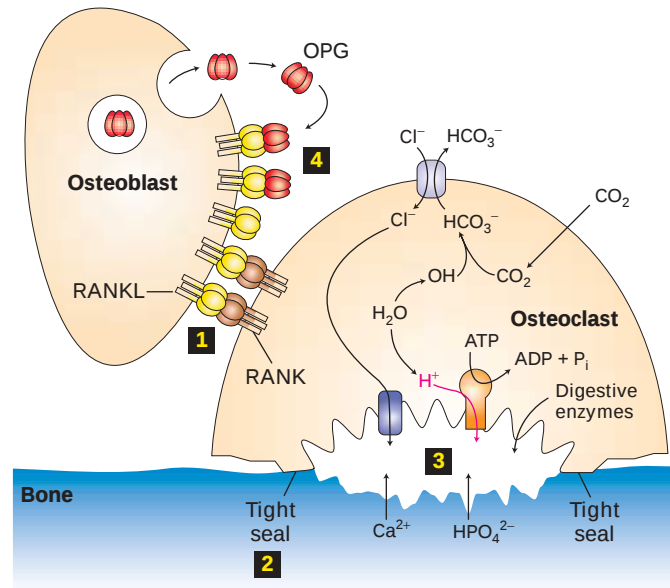
Another way of reducing the activity of cell-surface receptors is secretion of a protein that contains a hormone-binding segment but no signal-transducing activity. As might be expected, hormone binding to such proteins, called *decoy receptors*, reduces the amount of hormone available to bind to receptors capable of signaling. This type of regulation is important in controlling bone resorption, a complex physiological process that integrates several molecular mechanisms.

Net bone growth in mammals subsides just after puberty, but a finely balanced, highly dynamic process of disassembly (resorption) and reassembly (bone formation), called *remodeling*, goes on throughout adulthood. Remodeling permits the repair of damaged bones and can release calcium, phosphate, and other ions from mineralized bone into the blood for use elsewhere in the body.

Osteoclasts, the bone-dissolving cells, are a type of macrophage that contain highly dynamic integrin-containing adhesive structures, called *podosomes*, in the plasma membrane (see Figure 6-27). The $\alpha_v\beta_3$ integrin in podosomes is crucial to the initial binding of osteoclasts to the surface of bone, since antibodies that bind to and block the activity of this integrin block bone resorption. Following their initial adhesion to bone, osteoclasts form specialized, very tight seals between themselves and bone, creating an enclosed extracellular space (Figure 14-31). An adhered osteoclast then secretes into this space a corrosive mixture of HCl and proteases that resorbs the bone 3. Osteoblasts can suppress bone resorption by secreting osteoprotegerin (OPG). Binding of this decoy receptor to RANKL 4 blocks RANKL binding to RANK on osteoclasts and thus their activation. See the text for discussion. [Adapted from N. Takahashi et al., 1999, *Biochem. Biophys. Res. Comm.* 256:449.]

Bone resorption by osteoclasts is carefully regulated by cell-cell interactions with neighboring *osteoblasts*. These bone-forming cells secrete type I collagen, the major organic component of bones. Osteoblasts express a trimeric cell-surface signaling protein termed *RANKL* that is a member of the TNF- α superfamily of trimeric signaling proteins. RANKL is the ligand for *RANK*, a cell-surface receptor expressed by osteoclasts. Interaction of RANK with RANKL initiates multiple intracellular signaling pathways in osteoclasts, including the NF- κ B pathway that also is initiated by stimulation of TNF- α receptors (see Figure 14-28). Collectively, these signals induce the differentiation of osteoclasts and changes in their shape that promote tight binding to bone and thus bone resorption.

Osteoblasts also produce and secrete a soluble decoy receptor protein called *osteoprotegerin (OPG)*, named for its ability to “protect bone.” Secreted OPG binds to RANKL on



▲ FIGURE 14-31 Bone resorption and its regulation.

Osteoclasts initially bind to bone via integrin-mediated podosomes. The subsequent activation of an osteoclast by interaction with neighboring osteoblasts via the trimeric membrane proteins RANKL and RANK 1 induces cytoskeletal reorganization, leading to formation of a specialized tight seal with bone 2. The activated osteoclast secretes into the extracellular space generated by this seal a corrosive mixture of HCl and proteases that resorbs the bone 3. Osteoblasts can suppress bone resorption by secreting osteoprotegerin (OPG). Binding of this decoy receptor to RANKL 4 blocks RANKL binding to RANK on osteoclasts and thus their activation. See the text for discussion. [Adapted from N. Takahashi et al., 1999, *Biochem. Biophys. Res. Comm.* 256:449.]

the surface of osteoblasts, thereby preventing the RANKL-RANK interaction and inhibiting osteoclast activation and bone resorption (see Figure 14-31). Mice deleted for the OPG gene have weak, porous bones characteristic of excessive resorption. This finding supports the essential function of OPG in reducing bone resorption.



The rare hereditary disease *osteopetrosis*, marked by increased bone density, is due to abnormally low resorption. Far more common is *osteoporosis*, which is most prevalent among postmenopausal women. This metabolic disorder results from disproportionate bone resorption, leading to porous, less dense bones that are readily broken or fractured.

Many steroid hormones (e.g., estrogen, glucocorticoids), vitamin D, polypeptide hormones, and drugs influence bone metabolism by directly interacting with osteoblasts and altering the RANKL/RANK signaling system. Estrogen, for example, normally induces secretion of OPG and thus inhibits bone resorption. When estrogen is low, as it is in many post-

menopausal women, resorption increases and the bones weaken. It may be possible to develop new treatments for osteoporosis based on altering the signaling system that controls bone resorption. ■



Because they bind their ligands so tightly, soluble extracellular domains of cell-surface hormone receptors are finding increasing use as therapeutics. Many cell-surface receptors are oriented in the plasma membrane such that the C-terminal signal-transducing domain extends into the cytosol and the N-terminal ligand-binding domain extends into the extracellular space. With recombinant DNA techniques a stop codon can be placed in the cDNA encoding such a receptor so that translation in an appropriate expression system generates a truncated protein corresponding to the receptor's extracellular domain, which will be secreted and can function as a decoy receptor. For example, local increases in TNF- α are frequent in *rheumatoid arthritis*, an inflammatory joint disease. Injection of the recombinant-produced extracellular domain of the TNF- α receptor, which "soaks up" some of the excess TNF- α and reduces inflammation, is now one of the major therapies for severe cases of this disease. ■

KEY CONCEPTS OF SECTION 14.7

Down-Modulation of Receptor Signaling

- Endocytosis of receptor-hormone complexes and their degradation in lysosomes is a principal way of reducing the number of receptor tyrosine kinases and cytokine receptors on the cell surface, thus decreasing the sensitivity of cells to many peptide hormones.
- Bone resorption is triggered by binding of RANKL on osteoblasts to its receptor, RANK, on osteoclasts. RANKL/RANK signaling promotes tight adhesion of osteoclasts to bone and secretion of a bone-dissolving mixture of HCl and proteases by osteoclasts (see Figure 14-31).
- Osteoprotegerin, a decoy receptor for RANKL, inhibits osteoclast activation and bone resorption.
- The extracellular domains of many cell-surface receptors can be produced by recombinant DNA techniques and have potential as therapeutic decoy receptors. Already in use is such a decoy receptor for TNF- α , which binds excess TNF- α associated with rheumatoid arthritis and other inflammatory diseases.

PERSPECTIVES FOR THE FUTURE

The confluence of genetics, biochemistry, and structural biology has given us an increasingly detailed view of how signals are transmitted from the cell surface and transduced into

changes in cellular behavior. The multitude of different extracellular signals, receptors for them, and intracellular signal-transduction pathways fall into a relatively small number of classes, and one major goal is to understand how similar signaling pathways often regulate very different cellular processes. For instance, STAT5 activates very different sets of genes in erythroid precursor cells, following stimulation of the erythropoietin receptor, than in mammary epithelial cells, following stimulation of the prolactin receptor. Presumably STAT5 binds to different groups of transcription factors in these and other cell types, but the nature of these proteins and how they collaborate to induce cell-specific patterns of gene expression remain to be uncovered.

Conversely, activation of the same signal-transduction component in the same cell through different receptors often elicits different cellular responses. One commonly held view is that the duration of activation of the MAP kinase and other signaling pathways affects the pattern of gene expression. But how this specificity is determined remains an outstanding question in signal transduction. Genetic and molecular studies in flies, worms, and mice will contribute to our understanding of the interplay between different pathway components and the underlying regulatory principles controlling specificity in multicellular organisms.

Researchers have determined the three-dimensional structures of various signaling proteins during the past several years, permitting more detailed analysis of several signal-transduction pathways. The molecular structures of different kinases, for example, exhibit striking similarities and important variations that impart to them novel regulatory features. The activity of several kinases, such as Raf and protein kinase B (PKB), is controlled by inhibitory domains as well as by multiple phosphorylations catalyzed by several other kinases. But our understanding of how the activity of these and other kinases is precisely regulated to meet the cell's needs will require additional structural and cell biological studies.

Abnormalities in signal transduction underlie many different diseases, including the majority of cancers and many inflammatory conditions. Detailed knowledge of the signaling pathways involved and the structure of their constituent proteins will continue to provide important molecular clues for the design of specific therapies. Despite the close structural relationship between different signaling molecules (e.g., kinases), recent studies suggest that inhibitors selective for specific subclasses can be designed. In many tumors of epithelial origin, the EGF receptor exhibits constitutive (signal-independent) protein tyrosine kinase activity, and a specific inhibitor of this kinase (IressaTM) has proved useful in the treatment of several such cancers. Similarly, monoclonal antibodies or decoy receptors that prevent pro-inflammatory cytokines like IL-1 and TNF- α from binding to their cognate receptors are now being used in treatment of several inflammatory diseases such as arthritis.

Drugs that target other signal-transducing proteins may be useful in controlling their abnormal activities. One example is Ras, which is anchored to cell membranes by farnesyl

groups that are linked to Ras by farnesyl transferase. Inhibitors of this enzyme are being tested as therapeutic agents in cancers caused by expression of constitutively active Ras proteins. Detailed structural studies of the interaction between signal-transducing proteins offer exciting possibilities for designing new types of highly specific drugs. For instance, knowledge of the interface between the Sos and Ras proteins or between Ras and Raf could provide the basis of a drug that blocks activation of MAP kinase. As more signaling pathways become understood at a molecular level, additional targets for drug development will undoubtedly emerge.

KEY TERMS

activation lip 578	PI-3 kinase pathway 598
constitutive 574	presenilin 1 603
cytokines 580	protein kinase B 599
decoy receptors 606	PTEN phosphatase 600
erythropoietin 581	Ras protein 587
JAK-STAT pathway 581	receptor tyrosine kinases 578
kinase cascade 593	regulated intramembrane
MAP kinase 593	proteolysis 603
NF- κ B pathway 601	scaffold proteins 597
Notch/Delta pathway 601	SH2 domains 579
nuclear-localization	Smads 575
signal 576	TGF β superfamily 574
phosphoinositides 598	

REVIEW THE CONCEPTS

1. Binding of TGF β to its receptors can elicit a variety of responses in different cell types. For example, TGF β induces plasminogen activator inhibitor in epithelial cells and specific immunoglobulins in B cells. In both cell types, Smad3 is activated. Given the conservation of the signaling pathway, what accounts for the diversity of the response to TGF β in various cell types?

2. How is the signal generated by binding of TGF β to cell-surface receptors transmitted to the nucleus where changes in target gene expression occur?

3. Name three features common to the activation of cytokine receptors and receptor tyrosine kinases. Name one difference with respect to the enzymatic activity of these receptors.

4. The intracellular events that proceed when erythropoietin binds to its cell-surface receptor are well-characterized examples of cell-signaling pathways that activate gene expression. What molecule translocates from the cytosol to the nucleus after (a) JAK2 activates STAT5 and (b) GRB2 binds to the Epo receptor?

5. Once an activated signaling pathway has elicited the proper changes in target gene expression, the pathway must be inactivated. Otherwise, pathologic consequences may result, as exemplified by persistent growth factor pathway signaling in many cancers. Many signaling pathways possess intrinsic negative feedback by which a downstream event in a pathway turns off an upstream event. Describe the negative feedback that down-regulates signals induced by (a) TGF β and (b) erythropoietin.

6. GRB2 is an essential component of the epidermal growth factor (EGF) signaling pathway even though GRB2 lacks intrinsic enzymatic activity. What is the function of GRB2? What role do the SH2 and SH3 domains play in the function of GRB2? Many other signaling proteins possess SH2 domains. What determines the specificity of SH2 interactions with other molecules?

7. A mutation in the Ras protein renders Ras constitutively active (Ras^D). What is *constitutive activation*? How is constitutively active Ras cancer-promoting? What type of mutation might render the following proteins constitutively active: (a) Smad3; (b) MAP kinase; and (c) NF- κ B?

8. The enzyme Ste11 participates in several distinct MAP kinase signaling pathways in the budding yeast *S. cerevisiae*. What is the substrate for Ste11 in the mating factor signaling pathway? When a yeast cell is stimulated by mating factor, what prevents the induction of filamentation since Ste11 also participates in the MAP kinase signaling pathway induced by starvation?

9. Describe the events required for full activation of protein kinase B. Name two effects of insulin mediated by protein kinase B in muscle cells.

10. Describe the function of the PTEN phosphatase in the PI-3 kinase signaling pathway. Why is a loss-of-function mutation in PTEN cancer-promoting? Predict the effect of constitutively active PTEN on cell growth and survival.

11. Why is the signaling pathway that activates NF- κ B considered to be relatively irreversible compared to cytokine or receptor tyrosine kinase signaling pathways? Nonetheless, NF- κ B signaling must be downregulated eventually. How is the NF- κ B signaling pathway turned off?

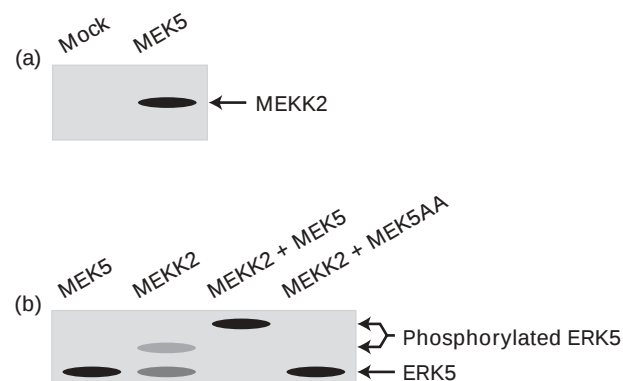
12. What biochemical reaction is catalyzed by the enzyme presenilin 1? What is the role of presenilin 1 in transducing the signal induced by the binding of Delta to its receptor? How are mutations in presenilin 1 thought to contribute to Alzheimer's disease?

13. What is a decoy receptor? How does the decoy receptor osteoprotegerin block bone resorption? Some tumors are characterized by excess production of platelet-derived growth factor (PDGF). How might a recombinant decoy receptor function as a chemotherapeutic agent for such a cancer?

ANALYZE THE DATA

G. Johnson and colleagues have analyzed the kinase cascade in which MEKK2, a MAP kinase kinase kinase, participates in mammalian cells. By a yeast two-hybrid screen (see Chapter 11), MEKK2 was found to bind MEK5, a MAP kinase kinase. To elucidate the signaling pathway transduced by MEKK2 in vivo, the following studies were performed in human embryonic kidney (HEK293) cells in culture.

a. HEK293 cells were transfected with a plasmid encoding recombinant, tagged MEKK2, along with a plasmid encoding MEK5 or a control vector that did not encode a protein (mock). Recombinant MEK5 was precipitated from the cell extract by absorption to a specific antibody. The immunoprecipitated material was then resolved by polyacrylamide gel electrophoresis, transferred to a membrane, and examined by Western blotting with an antibody that recognized tagged MEKK2. The results are shown in the figure below, part a. What information about the MEKK2 kinase cascade do we learn from this experiment? Do the data in part a of the figure prove that MEKK2 activates MEK5, or vice versa?



b. ERK5 is a MAP kinase previously shown to be activated when phosphorylated by MEK5. When ERK5 is phosphorylated by MEK5, its migration on a polyacrylamide gel is retarded. In the experiment shown in part b of the figure, HEK293 cells were transfected with a plasmid encoding ERK5 along with plasmids encoding MEK5, MEKK2, MEKK2 and MEK5, or MEKK2 and MEK5AA. MEK5AA is a mutant, inactive version of MEK5 that functions as a dominant-negative. Expression of MEK5AA in HEK293 cells prevents signaling through active, endogenous MEK5. Lysates of transfected cells were analyzed by Western blotting with an antibody against recombinant ERK5. From the data in part b of the figure, what can we conclude about the role of MEKK2 in the activation of ERK5? How do the data obtained when cells are cotransfected with ERK5, MEKK2, and MEK5AA help to elucidate the order of participants in this kinase cascade?

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