

CHAPTER

## 26

## Tyrosine Phosphorylation

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Nonreceptor tyrosine phosphatases are cytoplasmic and have regulatory sequences flanking the catalytic domain			

## TYROSINE PHOSPHORYLATION IN THE NERVOUS SYSTEM

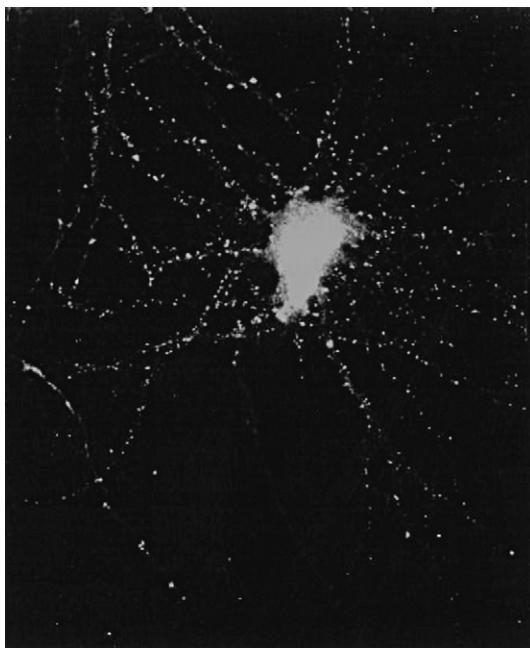
Protein phosphorylation is one of the most important mechanisms in the regulation of cellular function. Proteins can be phosphorylated on serine, threonine or tyrosine residues. Most phosphorylation occurs on serine and threonine (see Ch. 25), with less than 1% on tyrosine. This perhaps accounts for the late discovery of tyrosine phosphorylation, which was found first on polyoma virus middle T antigen in 1979 by Hunter and colleagues (Hunter & Eckhart, 2004). Since then, a plethora of tyrosine-phosphorylated proteins has been discovered. Originally, tyrosine phosphorylation was believed to be involved primarily in regulating cell proliferation, since many oncogene products and growth factor receptors are protein tyrosine kinases (PTKs). However, it has become clear that tyrosine phosphorylation is involved in regulating a variety of cellular processes. In fact, the nervous system contains a large variety of PTKs and protein tyrosine phosphatases (PTPs), and some

of these are exclusively expressed in neuronal tissues. Figure 26-1 shows the immunocytochemical staining of a cultured hippocampal neuron with a phosphotyrosine antibody (Lau & Huganir, 1995). It reveals the presence of tyrosine-phosphorylated proteins in the cell body as well as in synapses, suggesting that tyrosine phosphorylation may play a role in neuronal function. Furthermore, many neuronal processes are modulated by inhibitors of PTK or PTP, or by modification of the genes encoding these enzymes.

Tyrosine phosphorylation is regulated by the balance between the activities of PTKs and PTPs (Fig. 26-2). Phosphorylation of a tyrosine residue adds negative charges to its side chain and increases its size. These alterations may consequently trigger structural changes and potential functional modifications in the tyrosine-phosphorylated protein. In addition, the phosphorylated tyrosine and its surrounding amino acids may become a molecular adhesive that interacts with phosphotyrosine binding proteins, thereby changing its subcellular localization and functions.

## PROTEIN TYROSINE KINASES

Upon completion of the Human Genome Project, there were 518 unique genes that encoded kinases (Manning et al., 2002) with 90 unique genes that are identified to encode PTKs (Robinson et al., 2000) and another 48 kinases with homologies to PTKs (Manning et al., 2002). Among the authentic PTKs, 58 encode receptor protein tyrosine kinases (RPTKs), which can be categorized into 20 subfamilies (Fig. 26-3) and 32 encode nonreceptor protein tyrosine kinases (NRPTKs), which belong to 10 subfamilies (Fig. 26-4) (Blume-Jensen & Hunter, 2001). RPTKs are integral membrane proteins while NRPTKs are intracellular, although they may have lipid anchors (see below). The catalytic activity of each type of kinase is subject to regulation by extracellular stimuli and intracellular signaling.



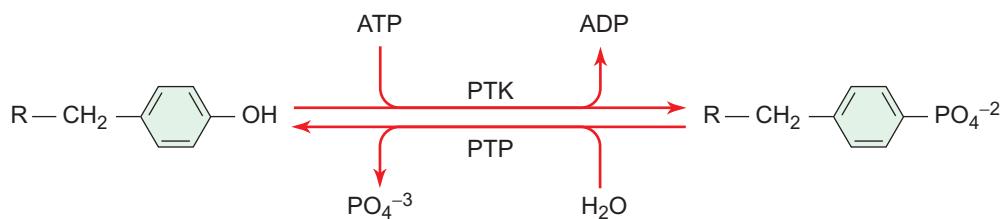
**FIGURE 26-1** Phosphotyrosine staining of a hippocampal neuron. A cultured rat hippocampal pyramidal neuron is stained with an antiphosphotyrosine antibody and detected by a secondary antibody conjugated to rhodamine. The staining reflects the presence of tyrosine-phosphorylated proteins throughout the neuron, including the cell body and synaptic regions.

Nonreceptor protein tyrosine kinases contain a catalytic domain, as well as various regulatory domains important for proper functioning of the enzyme

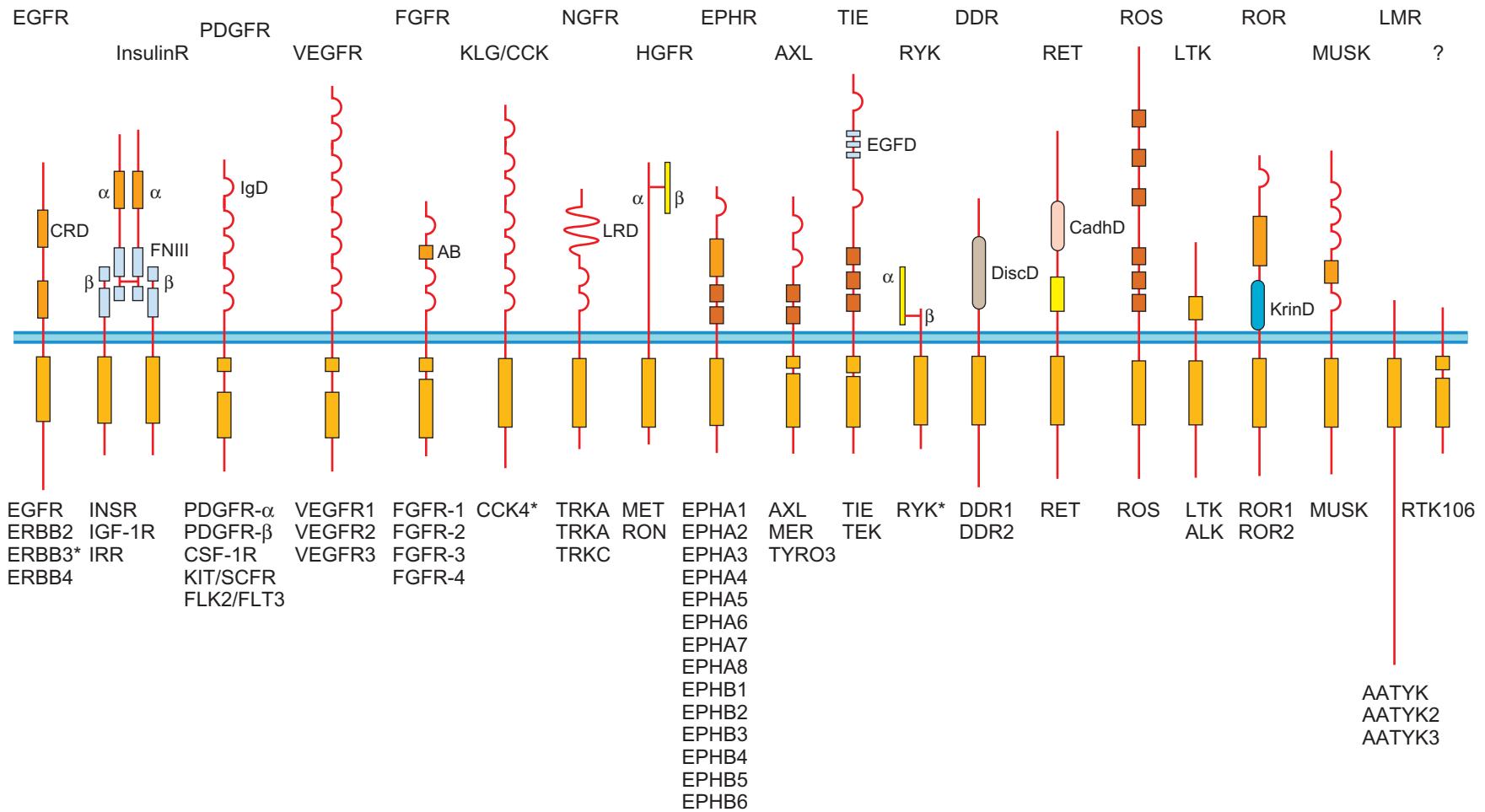
NRPTKs are found in the inner leaflet of the plasma membrane, cytosol, endosomal membranes and nucleus. These include the Src, Jak, Abl, Tec, Ack, Csk, Fak, Fes, Frk and Syk subfamilies (Fig. 26-4). Since a great deal is known about the structure and regulation of Src tyrosine kinase (Boggon et al., 2004), we will use it to illustrate the principles in NRPTK signaling; unique features in other subfamilies will be indicated when appropriate.

The typical catalytic domain of PTKs is about 250 amino acids in length and is by itself a functional enzyme. However, most, if not all, PTKs are larger and contain regulatory domains in addition to the catalytic domain. The regulatory domains restrain the catalytic domain and minimize basal kinase activity in the absence of stimulation. When stimulated, the regulatory domains relieve their restraints on the catalytic domain and allow kinase activation. The regulatory domains also direct the protein to specific subcellular targets or compartments, interact with potential substrates and assemble with other signaling molecules. The structural domains of the Src kinase family are, in order from the N-terminus: the SH4 (Src homology 4), SH3, SH2 and SH1 domains. SH1 is the catalytic domain; SH2 and SH3 are both molecular adhesives important for protein–protein interaction; whereas the SH4 domain, residing at the very N-terminus, is important for membrane attachment. Between the SH4 and the SH3 domains lies a region whose sequence varies considerably among different members of the Src family.

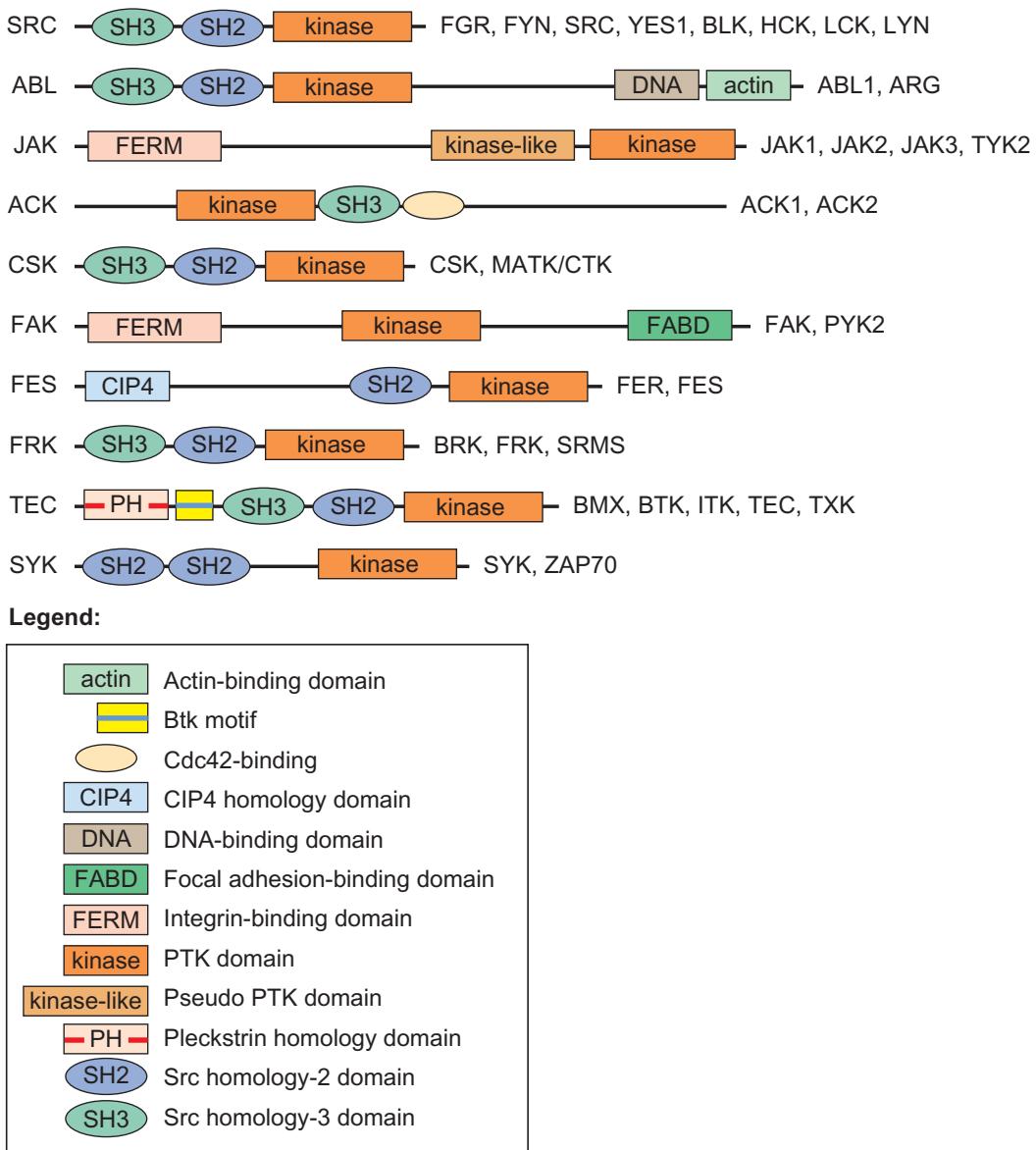
The SH4 domain, at the very N-terminus of the Src family kinases, contains a myristylation site critical for membrane localization of Src. N-myristylation is catalyzed co-translationally by *N*-myristoyl transferase. Following removal of the N-terminal methionine, myristate is transferred from myristoyl coenzyme A to the glycine residue at the second position. Although myristylation is necessary for anchoring Src to membrane, it is insufficient. The basic residues in the SH4 domain in Src and Blk interact with the negatively charged head groups of phospholipids and help to attach these kinases to the membrane. In other Src family members, palmitoylation of a cysteine residue in the SH4 domain secures their interaction with membrane. While myristylation is irreversible, electrostatic interaction and palmitoylation are reversible. For example, Ser17 in Src can be phosphorylated by protein



**FIGURE 26-2** Tyrosine phosphorylation and dephosphorylation. Protein tyrosine kinases (PTK) catalyze the transfer of the  $\gamma$ -phosphate group from ATP to the hydroxyl group of tyrosine residues, whereas protein tyrosine phosphatases (PTP) remove the phosphate group from phosphotyrosine. R represents the protein.



**FIGURE 26-3** Schematic structures of receptor protein tyrosine kinase (RPTK) families. RPTKs can be divided into different families according to the structural features in the extracellular domain. Following the extracellular domain are the transmembrane and intracellular domains; the latter contains the catalytic domain. (Adapted from Blume-Jensen & Hunter, 2001 with permission.)



**FIGURE 26-4** Nonreceptor protein tyrosine kinase (NRPTK) families. The family members are shown to the right and family name to the left of each NRPTK. Inset indicates the PTK catalytic domain and other domains for the regulation of localization and function. Kinases listed in bold have been associated with malignancies in humans and/or animal models. (Adapted from Blume-Jensen & Hunter, 2001 with permission.)

kinase A (PKA). Phosphorylation decreases the local positive charge and consequently dissociates Src from the negatively charged membrane. Similarly, palmitate can be removed from the cysteine residue to release Src family kinases from the membrane to the cytosol. An N-terminal pleckstrin homology (PH) domain in the Tec family tyrosine kinases anchors these kinases to membrane by binding specifically to phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P<sub>3</sub>) in membrane (see Ch. 23).

The unique domain following the SH4 domain from the N-terminus is a region with considerable variation in the amino acid sequences among different Src family members. This unique domain may be involved in protein–protein interactions, which may play a role in localization within the cell. For example, the unique region of Lck is linked to CD4 and

CD8, while those of Fyn and Lyn may be associated with the T- and B-cell antigen receptors.

The SH3 domain consists of approximately 60 amino acid residues and is C-terminal to the unique domain (Ingle, 2008; Mayer, 2001). Its five  $\beta$  strands form a globular structure whose N- and C-termini are in proximity to each other. SH3 domains mediate both intermolecular and intramolecular interactions, targeting short amino acid sequences that consist of specific proline-rich motifs in a left-handed helix–polyproline type II helix. Although SH3 domains were initially described in Src, this motif may be found in a variety of proteins where it mediates protein–protein interactions based on proline-rich domains (Kay et al., 2000; Macias et al., 2002). The specific proline-rich sequence recognized by a given SH3 domain may vary, but a typical SH3 domain ligand is composed of two prolines separated by

scaffolding residues (XP-X-XP) (reviewed in [Cesareni et al., 2002](#); [Macias et al., 2002](#)). It can bind to SH3 domain in either one of two opposite orientations, depending on the position of an additional positively charged residue in the peptide sequence. Type I ligands have a consensus sequence of RPLPPLP. Type II ligands adopt an inverted orientation and have a consensus sequence of  $\Phi$ PPLPXR, where  $\Phi$  represents a hydrophobic amino acid. Small peptide ligands have a low affinity for the SH3 domain ( $5\text{--}50\mu\text{mol/l}$ ) and low sequence specificity. Interaction between SH3 domains and proline-rich sequences within proteins confers higher affinity and specificity. Despite the presence of consensus SH3 binding sequences, variations from this central theme do exist for SH3 domains from different members of the Src family tyrosine kinases. The neuronal form of Src contains a six-amino-acid insert within the SH3 domain. This insertion appears to decrease the affinity of the SH3 domain for known ligands since most of the SH3 ligands fail to interact with the neuronal form of Src. This insertion may change the specificity of the Src SH3 domain for neuronal targets and increase its activity by reducing intramolecular interaction.

The SH2 domain is about 100 amino acid residues in length and lies between the SH3 and SH1 domains ([Filippakopoulos et al., 2009](#); [Schlessinger & Lemmon, 2003](#)). This domain interacts with phosphorylated tyrosine residue in a sequence-specific manner; thus this type of protein–protein interaction is directly regulated by tyrosine phosphorylation (reviewed in [Machida & Mayer, 2005](#); [Schlessinger & Lemmon, 2003](#)). As with SH3 motifs, a wide range of proteins may contain SH2 motifs, where they mediate binding to specific phosphotyrosines in target proteins ([Machida & Mayer, 2005](#); [Pawson, 2004](#)). SH2 domains contain two binding pockets for their ligands. One of these pockets contains a positively charged arginine residue, which interacts with the negatively charged phosphotyrosine residue. The other pocket interacts with three to five amino acids C-terminal to the phosphotyrosine and plays a major role in determining substrate specificity. However, amino acid residues N-terminal to the phosphotyrosine have also been shown to contribute to SH2 domain binding. The affinity of an SH2 domain for its interacting phosphotyrosine peptide is in the nanomolar range. Removal of the phosphate group from the peptide reduces the affinity for the peptide by about three orders of magnitude. Like the SH3 domain, the N- and C-termini of the SH2 domain are proximal to each other; this juxtaposition allows the formation of a protruding globular structure without significant disruption of the structure of the parent molecule and, importantly, exposes the ligand-binding surface. Compared to SH3 domain ligands, there is less of a consensus peptide sequence among SH2 ligands ([Table 26-1](#)). SH3 and SH2 domains are found in a variety of signaling molecules ([Pawson & Kofler, 2009](#)) as depicted in [Figure 26-5](#).

The SH1 domain is the catalytic domain ([Boggon et al., 2004](#); [Roskoski, 2004](#)). The crystal structures of Src-family tyrosine kinases have been solved ([Sicheri, Moarefi et al., 1997](#); [Xu, et al., 1997](#)). In general, the catalytic (SH1) domain has an overall bilobal structure, with a small N-terminal lobe and a large C-terminal lobe. The N-lobe contains a glycine-rich sequence, GXGXXGXV, and an invariable downstream lysine for ATP binding. Mutation of this lysine often leads to loss of catalytic activity. The C-lobe contains a glutamate residue that catalyzes transfer of the phosphate group from ATP

**TABLE 26-1** Specificity and Affinity of SH2, Phosphotyrosine Binding (PTB) and SH3 Domains

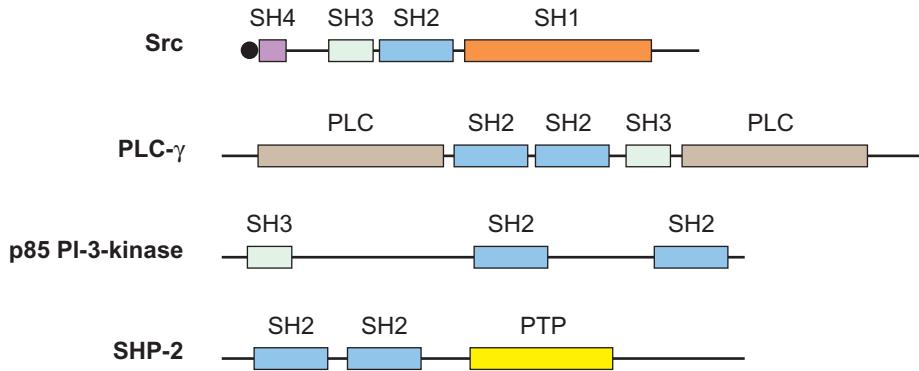
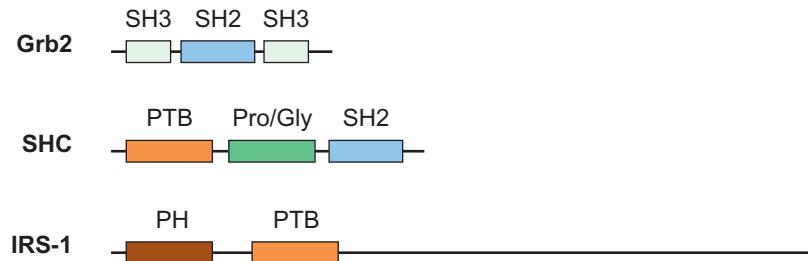
Domains	Consensus sequence of ligands	$K_D$
SH2	pYR <sub>1</sub> R <sub>2</sub> R <sub>3</sub>	1–10 nmol/l
PTB	$\Phi$ NPXpY	25–100 nmol/l
SH3	RPLPPLP, $\Phi$ PPLPXR	5–50 $\mu\text{mol/l}$

The consensus sequences of the ligands interacting with SH2, PTB and SH3 domains are listed with their dissociation constants. Both SH2 and PTB domains bind to phosphotyrosine in a sequence-specific manner. Tyrosine dephosphorylation can reduce the affinity of the SH2 domain to its ligand by 1,000-fold. R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> represent variable amino acids depending on the specific SH2 domain involved. X represents any amino acid.  $\Phi$  represents a hydrophobic amino acid; pY, phosphotyrosine.

to tyrosine. ATP fits in the cleft between the two lobes with its  $\gamma$ -phosphate pointing outward while the substrate peptide interacts at the cleft opening. There is considerable flexibility in the relative orientation of these two lobes. The active conformation of the kinase allows the proper alignment of the ATP terminal phosphate with the substrate tyrosine residue. However, the two lobes of an inactive kinase are swung apart so that the ATP terminal phosphate is not aligned with the substrate. [Figure 26-6A](#) shows the X-ray crystallographic analysis of Hck ([Sicheri et al., 1997](#)).

Under physiological conditions, NRPTKs are highly specific in directing tyrosine phosphorylation toward appropriate substrates. This specificity relies on the intrinsic predilection of the catalytic domain towards specific amino acid sequences within protein substrates and may be enhanced through docking elements in the target protein that may interact with the SH3 or SH2 domains ([Roskoski, 2004](#)). For example, phosphorylation of the tau microtubule associated protein by Fyn is greatly enhanced by docking of the enzyme to a proline rich domain near the microtubule binding repeats ([Lee, 2005](#)). In addition, noncatalytic domains—e.g., SH2, SH3 and PH (pleckstrin homology) domains of NRPTKs—distribute these kinases to the subcellular region where appropriate substrates are in proximity or abundance, thus favoring phosphorylation of these proteins rather than other substrates ([Ingleby, 2008](#)).

A major regulatory domain of Src is in the region C-terminal of the SH1 domain. It contains a tyrosine residue (Y527 in Src; see Glossary for single letter code for amino acids) critical for modulating kinase activity (reviewed in [Cole, Shen et al., 2003](#)). Under basal conditions, Y527 is phosphorylated by another NRPTK, C-terminal Src kinase (Csk). The phosphorylated Y527 interacts with the SH2 domain from the same molecule. This intramolecular interaction favors and is further stabilized by association of Src SH3 domain with the polyproline helix in the linker region between SH1 and SH2 domains. As a result of these interactions, a critical acidic residue is pushed away from the active site, resulting in inhibition of Src kinase activity. Mechanisms that relieve these intramolecular constraints can activate Src. One of these mechanisms is by dephosphorylation of Y527 and subsequent disruption of the two intramolecular interactions mentioned above. The initial Src kinase activity allows autophosphorylation of Y416 in the activation loop, additional conformational change and maximal

**Group 1: Enzymes****Group 2: Adaptors**

**FIGURE 26-5** SH2, SH3 and phosphotyrosine binding (PTB) domain-containing signaling proteins. SH2, SH3 and PTB domains are important molecular ‘adhesives’. These domains are found in enzymes and play important roles in the regulation of enzyme function. They also are found in proteins lacking any apparent catalytic domain, in which case they may serve as adaptor proteins, assembling signal-transduction complexes. The PTB domain of insulin receptor substrate-1 (IRS-1) shares a low degree of homology with the one on SHC. The black circle indicates a myristic acid moiety. *PLC*, phospholipase C; *PI3-kinase*, phosphatidylinositol-3-kinase; *PTP*, protein tyrosine phosphatase; *PH*, pleckstrin homology; *SHP*, SH2-containing PTP; *Grb*, growth-factor-receptor-binding protein.

enzyme activity subsequently. This model nicely accounts for some earlier observations. For example, v-Src, the tumorigenic form of Src that lacks the negative regulatory domain containing Y527, has very high basal activity. Mutation of Y527 to phenylalanine can increase Src activity. In light of the opposing effects of phosphorylation of Y527 and Y416 on Src kinase activity, different PTPs differentially regulate its enzyme activity. Dephosphorylation of Y527 catalyzed by the leukocyte common antigen (CD45) or an SH2 domain-containing PTP (SHP-2) activates Src, while dephosphorylation of Y416 by another phosphatase (SHP-1) inactivates it (Chong & Maiese, 2007). Figure 26-6B shows the regulatory mechanisms for Src family tyrosine kinases.

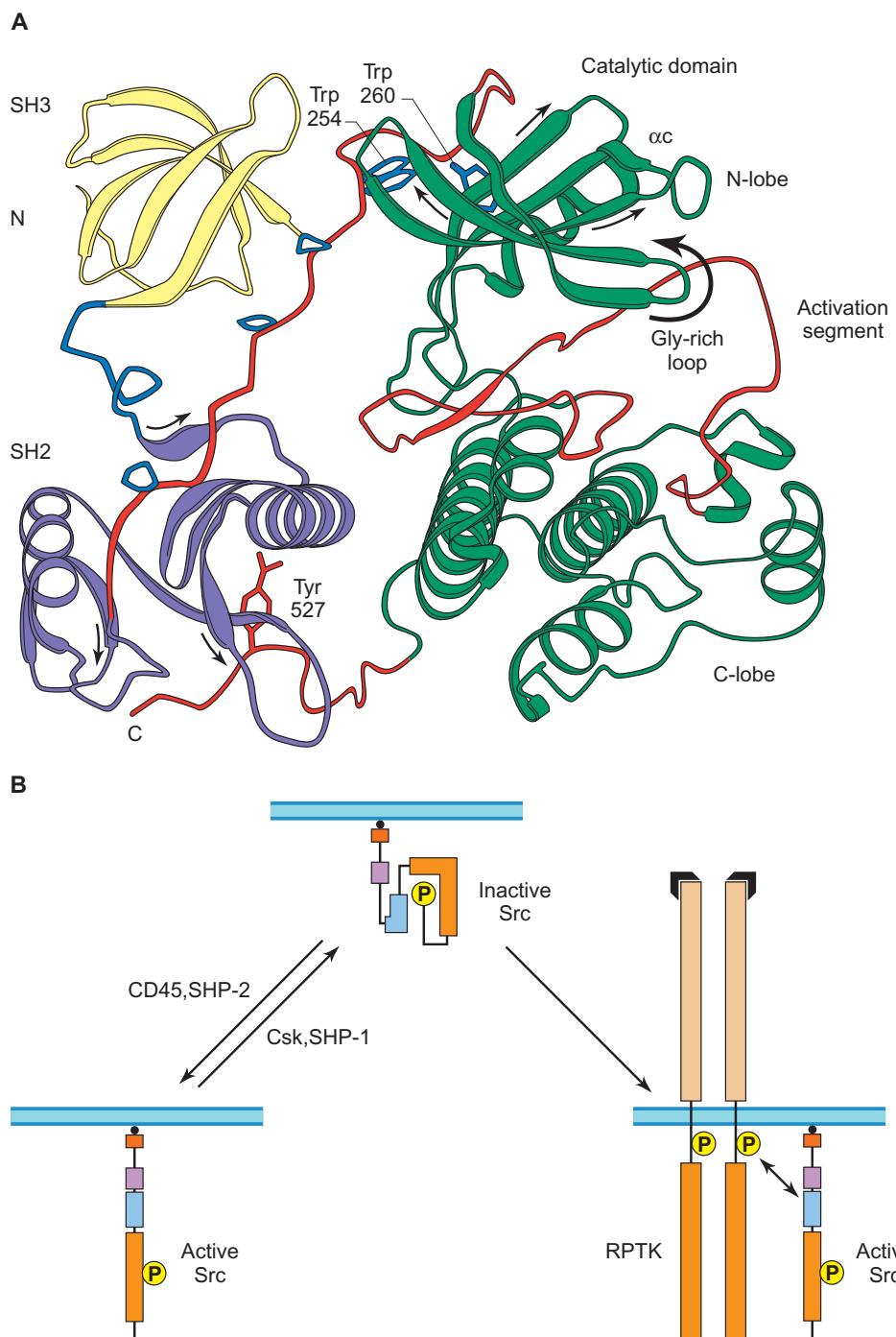
The key to activation of Src family kinases is the disruption of intramolecular interactions and relief of autoinhibition. In addition to dephosphorylation of Y527 by PTPs, the SH2-phosphoY527 bond can be disrupted by competition with a high-affinity phosphotyrosine ligand. For example, activation of the platelet-derived growth factor receptor (PDGFR) results in autophosphorylation of a number of tyrosine residues, including Y579 in the juxtamembrane region. This phosphotyrosine-containing peptide sequence interacts with the Src SH2 domain and displaces the negative regulatory domain, resulting in Src activation. The displaced Src C-terminal tail is now accessible to PTPs, allowing prolonged activation of Src despite a transient autophosphorylation of the PDGFR. A

similar mechanism applies to activation of Src by autophosphorylated Fak (Fig. 26-6B). Although NRPTKs do not possess an extracellular domain from which they can directly communicate with the extracellular world through this mechanism, they are able to respond to the extracellular stimulation by ‘borrowing’ extracellular domains from transmembrane proteins that interact with the NRPTKs.

In addition, the phosphotyrosine moiety (Y402) on a proline-rich tyrosine kinase (PYK2, a NRPTK) can apparently compete with phosphoY527 for interaction with SH2 domain on Src and lead to Src activation. PYK2 is a member of the Fak family. It is highly expressed in the nervous system. Its activation depends on both cell adhesion and the presence of calcium or PKC activation. Therefore, certain G protein-coupled receptors (GPCRs) that release calcium and activate PKC may play a role in the regulation of PYK2 and Src activities. (See Ch. 21 for detailed discussion of GPCRs.)

**Receptor protein tyrosine kinases consist of an extracellular domain, a single transmembrane domain and a cytoplasmic domain**

The cytoplasmic domain is composed of a juxtamembrane region, one or two catalytic domains and a C-terminal tail. Like the NRPTKs, the structures outside the catalytic domain are



**FIGURE 26-6** (A) Crystal structure of the Src family kinase Hck. The catalytic domain of Src family tyrosine kinases consists of an N-terminal ATP-binding lobe and a C-terminal substrate-binding lobe. There is considerable flexibility in the relative orientation of these two lobes. The active conformation allows the proper alignment of the ATP terminal phosphate, the substrate and a catalytic glutamate residue (Glu310). However, the two lobes of an inactive kinase are swung apart, disrupting proper alignment. (With permission from Sicheri et al. (1997)) (B) Regulation of Src activity. Src activity can be regulated in a number of ways. In the inactive state, phosphorylated Y527 interacts with the SH2 domain. In addition, the SH3 domain binds the linker region between the SH1 and SH2 domains. These two intramolecular interactions pull the two lobes of the catalytic domain apart and inactivate the kinase. Therefore, dephosphorylation of Y527 by protein tyrosine phosphatases, e.g. CD45 and SHP-2, activates the kinase. Phosphorylation of Y527 by Csk inactivates the kinase. Inactivation is also achieved by dephosphorylating the autophosphorylation site Y416 by SHP-1. Alternatively, Src can be activated by binding to an autophosphorylated receptor protein tyrosine kinase (RPTK), such as the platelet-derived growth factor receptor. The RPTK autophosphorylation site binds to the Src SH2 domain and thus disrupts the inhibitory intramolecular interactions and activates the kinase. Association of Src with tyrosine-phosphorylated Fak activates the kinase in a similar fashion.

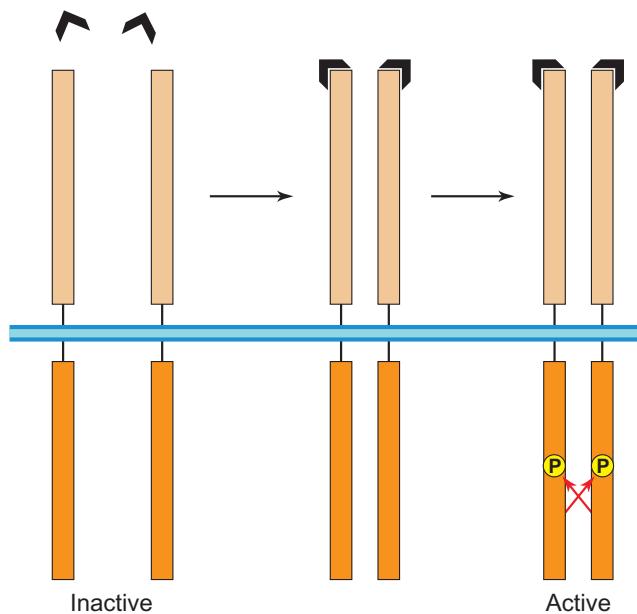
critical for the proper functioning of these enzymes. RPTKs can be classified into at least 20 families (Pawson, 2002; Robinson et al., 2000), including many of particular relevance for the nervous system, such as the TRK family for neurotrophins, epidermal growth factor receptor family (EGFR), platelet-derived growth factor receptor family (PDGFR), fibroblast growth factor receptor family (FGFR), insulin receptor (INSR), erythropoietin-producing hepatocellular receptor or Ephrin family (EphR), vascular endothelial growth factor receptor (VEGF) and muscle specific kinase (MUSK) (Fig. 26-3). (See also Ch. 29.)

The extracellular domains of RPTKs can be composed of different structural motifs and are quite diverse. For instance, the EGFR extracellular domain contains two cysteine-rich regions. The PDGFR extracellular domain consists of five immunoglobulin-like repeats. Other domains found in the extracellular region of RPTKs include fibronectin III repeats, kringle domains and leucine-rich motifs. The extracellular domains of members of the insulin receptor subfamily contain disulfide bridges responsible for the formation of a heterotetrameric structure ( $\alpha_2\beta_2$ ). These extracellular domains carry the binding sites for RPTK ligands, which range from soluble factors, extracellular matrix proteins, to surface proteins expressed on adjacent cells. The extracellular domain may also be involved in the dimerization of RPTKs, a process critical for the activation of intrinsic tyrosine kinase activity. The transmembrane domain in the RPTK is a hydrophobic segment of 22–26 amino acids inserted in the cell membrane. It is flanked by a proline-rich region in the N-terminus and a cluster of basic amino acids in the C-terminus. This combination of structures secures the transmembrane domain within the lipid bilayer. There is a low degree of homology in the transmembrane domain, even between two closely related RPTKs, suggesting that the primary sequence contains little information for signal transduction. The cytoplasmic domain primarily consists of the catalytic domain and various autophosphorylation sites that regulate catalytic function and serve as docking sites for SH2 domain-containing proteins. The protein kinase catalytic domains of RPTKs are highly conserved and similar in structure to those of the NRPTK (see above) (Robinson et al., 2000).

### RPTK Activation

The activity of RPTKs is normally suppressed in their quiescent state. This suppression is due to the numerous loose and unstructured conformations of the activation loop (A loop) within the catalytic domain; the majority of these conformations interfere with substrate and ATP binding. However, a subset of these conformations is amenable to binding of substrate and ATP, resulting in activation of the RPTKs. Phosphorylation of the tyrosine residue(s) in the A loop shifts the equilibrium towards the active conformation. Because of steric hindrance, RPTK catalytic domains appear to be unable to autophosphorylate tyrosine residue(s) in the A loop within the same molecule; rather *trans-autophosphorylation* between two different catalytic domains is necessary for their activation. As a consequence, ligand-induced dimerization is an important step in the activation of RPTKs (Fig. 26-7).

Different RPTKs use different mechanisms to achieve dimerization and *trans-autophosphorylation*. PDGF is a dimer consisting of subunits A and B in different combinations (AA, BB and AB). These dimeric PDGF ligands can physically

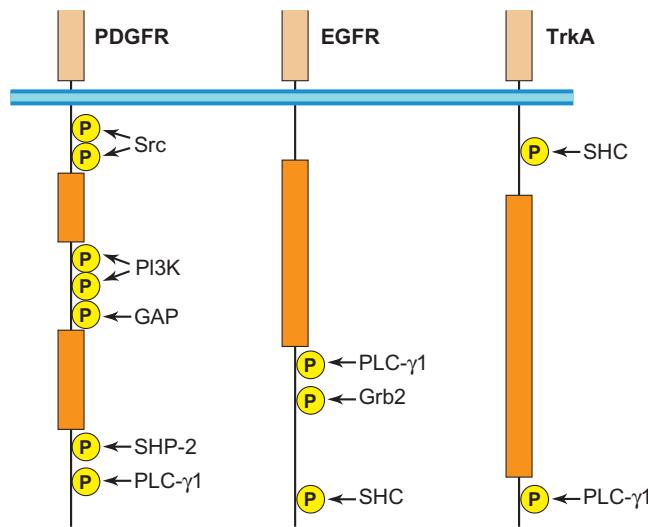


**FIGURE 26-7** Activation of receptor protein tyrosine kinase (RPTK) by dimerization and transphosphorylation. Autophosphorylation is a key step in the activation of PTKs. To achieve autophosphorylation, some PTKs dimerize and transphosphorylate each other. Ligand binding induces dimerization of RPTKs so that the catalytic domains are in proximity for transphosphorylation. The autophosphorylated RPTK, thus, is activated. Some nonreceptor protein tyrosine kinases (NRPTKs) can be activated without dimerization (see Fig. 26-5B). However, others, such as Jak/Tyk kinases, are activated by transphosphorylation. Jak/Tyk tyrosine kinases are associated constitutively with transmembrane proteins gp130 and leukemia-inhibitory factor receptor (LIFR) $\beta$ . Extracellular ciliary neurotrophic factor (CNTF) induces the formation of a quaternary complex consisting of CNTF, LIFR $\beta$ , gp130 and another membrane protein known as CNTF receptor  $\alpha$ . Consequently, the associated Jak/Tyk are dimerized, allowing transphosphorylation and activation.

and directly cross-link two PDGFRs because of their bivalence. In contrast, the EGFRs binding to their monomeric ligands may induce a conformational change in the extracellular domain that would subsequently associate with each other. Interestingly, the INSR consists of two catalytic domains already in proximity to each other even in their quiescent state due to their cross-linkage by intermolecular disulfide bonds in the extracellular domains (Fig. 26-3). In this case, the low basal kinase activity in the absence of ligand may be maintained by a suboptimal orientation or distance of the active sites to the A loop. Ligand binding may reorient or further reduce the distance between the catalytic domains resulting in *trans-autophosphorylation* and activation.

### RPTK Inactivation

Once activated by their ligands, surface RPTKs with their bound ligands are typically rapidly internalized and degraded. These processes quickly terminate the action of the ligands. The intrinsic tyrosine-kinase activity appears to be important for both internalization and degradation. For example, mutant EGFR without the lysine for ATP binding is neither internalized nor degraded in a mouse fibroblast cell line,



**FIGURE 26-8** Recruitment of different signaling molecules to different tyrosine-phosphorylation sites on receptor protein tyrosine kinases (RPTKs). RPTKs usually are autophosphorylated on several different sites. Each tyrosine-phosphorylation site has a unique sequence for interaction with specific signaling molecules containing SH2 or phosphotyrosine-binding (PTB) domains. This provides specificity and diversity for RPTK signaling. *PDGFR*, platelet-derived growth factor receptor; *EGFR*, epidermal growth factor receptor; *PLC*, phospholipase C; *GAP*, GTPase-activating protein; *Grb2*, growth factor receptor binding protein; *PI3K*, phosphatidylinositol-3-kinase; *SHC*, SH2-containing protein; *SHP-2*, SH2-domain containing tyrosine phosphatase.

NIH3T3 cells. In certain situations, internalized RPTKs continue to mediate signal transduction mechanisms. For instance, in sympathetic neurons, internalized TrkA is transported from the axon terminal to the cell body where it influences signal transduction cascades that affect gene expression (Cosker et al., 2008) (see Chs 8 and 29).

### Tyrosine Phosphorylation of RPTKs

Phosphorylation of specific tyrosine residues on the RPTKs can recruit SH2 domain-containing signaling molecules to the site of action. These signaling molecules include phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1), phosphatidylinositol-3-kinase (PI-3-kinase), growth factor receptor-binding protein 2 (Grb2), SH2-containing protein (SHC) and Src. Each signaling molecule recognizes a specific tyrosine phosphorylation site on the RPTK, as indicated in Figure 26-8. Recruitment of PLC- $\gamma$ 1 and PI-3-kinase to the membrane brings the enzymes to their substrates in the lipid bilayer. PLC- $\gamma$ 1 is phosphorylated and activated by RPTKs, whereas association of PI-3-kinase with the autophosphorylated RPTKs induces its activity allosterically. PLC- $\gamma$ 1 catalyzes formation of diacylglycerol and inositol trisphosphate ( $IP_3$ ) from phosphatidylinositol-4,5-bisphosphate ( $PIP_2$ ) (see also Ch. 20). Diacylglycerol activates PKC, while  $IP_3$  triggers calcium release from intracellular stores. PI-3-kinase incorporates a phosphate group in the 3 position on the inositol ring of phosphatidylinositol phospholipids. One of the products, PI-3,4-P<sub>2</sub>, activates Akt kinase and promotes neuronal survival. PTEN, a phosphatase with activity towards both phospholipids and protein,

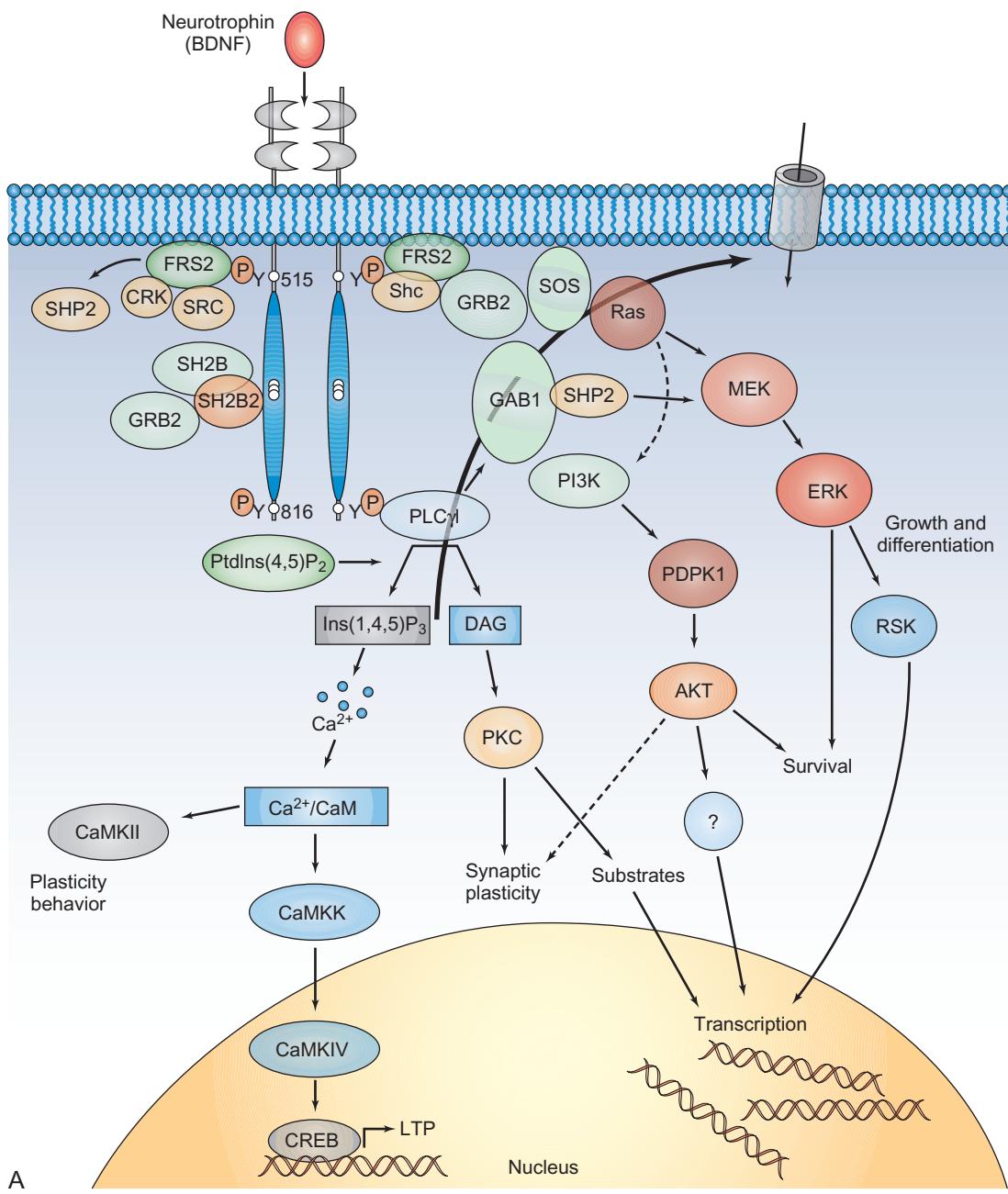
is an important mediator of this aspect of RPTK signaling PIP<sub>3</sub> (Abounader, 2009); (see Ch. 23).

Recruitment of Grb2 to the membrane activates the MAPK pathway. Grb2 is an adaptor molecule carrying one SH2 and two SH3 domains (Fig. 26-5). The Grb2 SH2 domain recognizes the tyrosine-phosphorylated moiety on certain RPTKs, such as EGFR (Fig. 26-8), and anchors itself to these membrane-spanning proteins. The Grb2 SH3 domains interact with son of sevenless (SOS), a guanine nucleotide exchange protein. SOS stimulates the release of GDP and subsequent binding of GTP to the membrane-bound, low-molecular weight, GTP-binding protein Ras (see Ch. 21). GTP-bound Ras interacts with and translocates the serine-threonine protein kinase Raf to the plasma membrane, where Raf becomes activated. Activated Raf phosphorylates MEK, which in turn stimulates MAPK. One of the substrates of MAPK is p90Rsk. Both MAPK and p90Rsk translocate to the nucleus after phosphorylation, where they phosphorylate and activate transcription factors, such as serum-responsive factor (SRF), T-cell-specific transcription factor and cAMP responsive element-binding protein, ultimately altering gene expression.

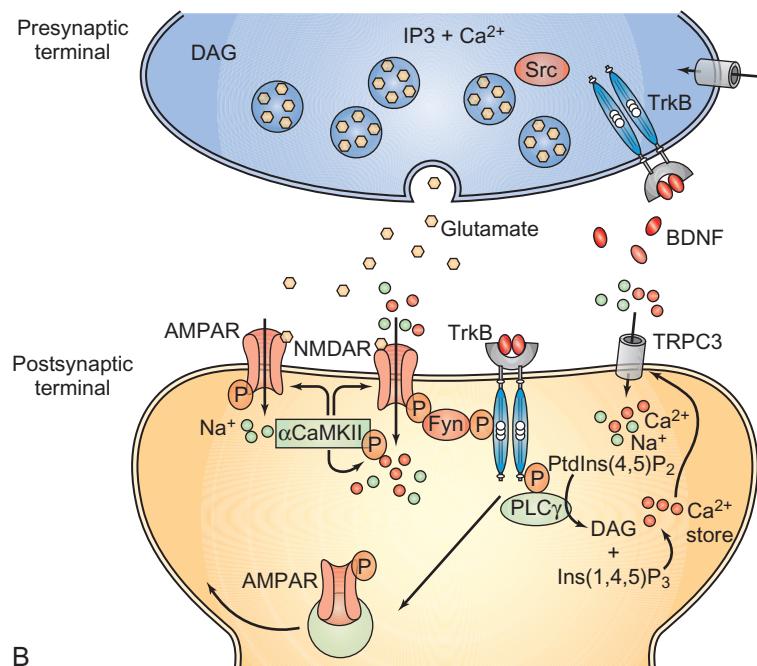
Some RPTKs cannot interact directly with Grb2. They activate the MAPK pathway with the help of an adaptor molecule, such as SHC or insulin receptor substrate-1 (IRS-1). SHC contains a 160-amino-acid phosphotyrosine-binding domain (PTB) that recognizes an autophosphorylation site on some RPTKs (Schlessinger & Lemmon, 2003). Unlike the SH2 domain, which recognizes the sequence C-terminal of the phosphotyrosine residue, PTB domains recognize amino acid residues upstream of phosphotyrosine. The consensus sequence for interaction with the PTB domain of SHC is  $\Phi$ NPXpY, where  $\Phi$  is a hydrophobic amino acid and X is any amino acid. IRS-1 contains a putative PTB domain that shares a low degree of homology with the PTB domain in SHC. After docking to the phosphotyrosine sites on RPTKs, both IRS-1 and SHC are in turn phosphorylated by their host RPTKs. Tyrosine phosphorylation of these adaptor molecules creates a binding site for Grb2, leading to activation of the MAPK cascade as mentioned above. Similarly, a PTP, SHP-2, plays a similar adaptor role in mediating the activation of MAPK pathway by RPTKs (Chong & Maiese, 2007); (see below). RPTKs that interact with a PTB-binding domain-containing adaptor molecule include the nerve growth factor receptor (TrkA), EGFR, INSR and insulin-like growth factor (IGF)-1 receptor. Figure 26-9 shows the role of SHC in RPTK-mediated gene expression.

### PROTEIN TYROSINE PHOSPHATASES

The low level of tyrosine phosphorylation in cells is probably attributable to the high specific activity of PTPs, which is about 10–1,000 times that of PTKs. The first PTP purified was PTP1B from human placenta. Based on the sequence of PTP1B, other PTPs were isolated very rapidly using molecular biological techniques, including polymerase chain reaction and low-stringency hybridization. Recent advances in sequencing the human genome have revealed more PTPs; it is estimated that there are in total over 100 (Tonks, 2006), making PTPs the largest group of phosphatase genes and comparable in number to



**FIGURE 26-9 Major signal-transduction mechanisms in TrkB/BDNF-mediated neuronal survival and differentiation.** (A) BDNF binds to TrkB and induces autophosphorylation on three different sites in the intracellular domain. One site interacts with SHC, which eventually leads to the activation of mitogen-activated protein kinase (MAPK) and transcription. TrkB also induces tyrosine phosphorylation of suc-associated neurotrophic factor-induced target (SNT) via a mechanism independent of all known signaling mechanisms. MAPK-induced transcription and SNT tyrosine phosphorylation are believed to be important in neuronal differentiation. TrkB autophosphorylation also activates phosphatidylinositol-3-kinase (PI3K) by recruiting it to the membrane. PI3K generates PI-3,4,5-trisphosphate from PI-4,5-bisphosphate. PI-3,4,5-trisphosphate is hydrolyzed to yield PI-3,4-bisphosphate, which activates Akt by translocating it to the membrane. Activated Akt can promote neuronal survival. Grb2, growth factor receptor binding protein, promotes survival and growth of neurons and other cells through Ras or GRB-associated binder 1 (GAB1). Phosphorylation at Y816 recruits and activates phospholipase C $\gamma$ 1 (PLC $\gamma$ 1), resulting in production of inositol-1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>) and diacylglycerol (DAG). Whereas DAG stimulates protein kinase C (PKC) isoforms, (Ins(1,4,5)P<sub>3</sub>) promotes the release of Ca<sup>2+</sup> from internal stores and subsequent activation of Ca<sup>2+</sup>/calmodulin (Ca<sup>2+</sup>/CaM)-dependent protein kinases (CaMKII, CaMKK and CaMKIV). These pathways can be functionally separated, so mutation at Y816 affects the PLC $\gamma$ 1 pathway, but not Ras/MAPK or PI3K pathways that are regulated by SHC. BDNF, brain-derived neurotrophic factor; FRS2, fibroblast growth factor receptor substrate 2; GRB2, growth factor receptor-bound protein 2; PDPK1, 3-phosphoinositide-dependent protein kinase 1; PtdIns(4,5)P<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; RSK, ribosomal protein S6 kinase; SHP2, SRC-homology phosphatase 2; SOS, son of sevenless.



**FIGURE 26-9** (Continued) (B) TrkB signaling affects both pre- and postsynaptic function. On the presynaptic side, BDNF binding activates Src-family NRPTK, which triggers endocytosis of TrkB-BDNF complex for return to the neuronal cell body by a retrograde transport of a signaling endosome (see Fig. 7-7). In addition, BDNF can increase Ca<sup>2+</sup> influx through transient receptor potential (TRPC) channels and enhance neurotransmitter release. On the postsynaptic side, there are functional interactions between TrkB signaling and glutamate receptors as well as TRP channels. NMDAR function is enhanced by activation of the Src family NRPTK, Fyn, which is activated by BDNF binding to TrkB. Fyn mediated phosphorylation of NMDARs increases the open probability of the NMDAR ion channel. Concurrently, BDNF binding to TrkB can activate PLC $\gamma$ , which leads to Ins(1,4,5)P<sub>3</sub>-dependent Ca<sup>2+</sup> store depletion, which activates influx of Ca<sup>2+</sup> and Na<sup>+</sup> through TRPC3 as well as altering the expression and trafficking of AMPARs. Ca<sup>2+</sup> influx through NMDARs activates  $\alpha$ -Ca<sup>2+</sup>/calmodulin-dependent protein kinase II ( $\alpha$ CaMKII) and contributes to the long-term potentiation through positive regulation of NMDARs and AMPARs. Activation of postsynaptic signaling pathways leads to increased release of BDNF from the postsynaptic cell, leading to increased synaptic strength and promoting survival of the presynaptic neuron through retrograde signaling. DAG, diacylglycerol; PtdIns(4,5)P<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate. Adapted from L. Minichiello, 850 (2009) figures 1 and 2.

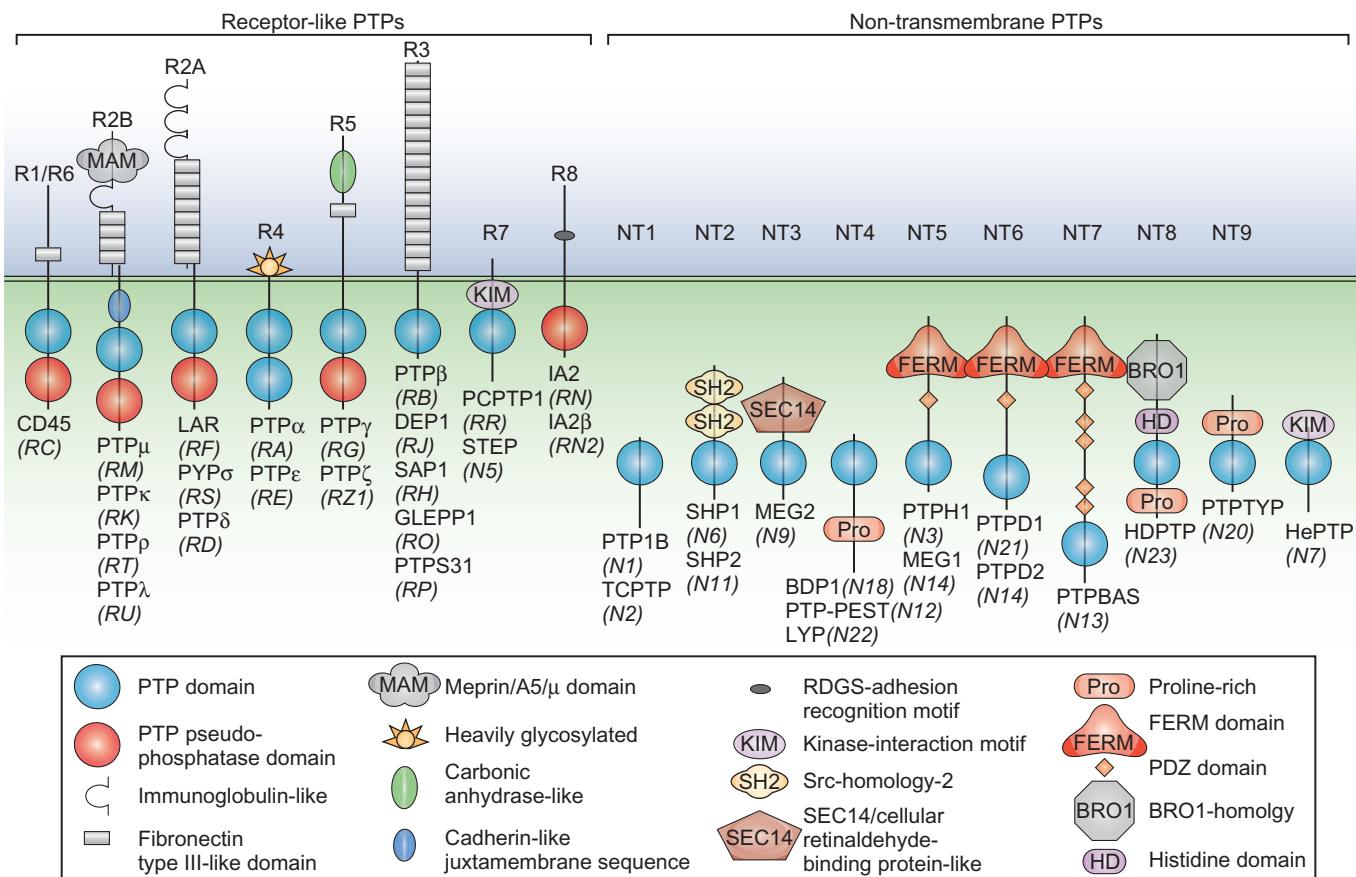
90 genes identified as PTKs (Manning et al., 2002). In contrast, the number of serine/threonine phosphatases is much less than the number of serine/threonine kinases (see Ch. 25). PTPs can be divided into classical PTPs (approximately 37 genes), which are specific for phosphotyrosine, and dual-specificity phosphatases (DSPs), which recognize both phosphotyrosine and phosphoserine/threonine (65 genes); (see also Ch. 25). Like PTKs, phosphotyrosine-specific PTPs consist of receptor protein tyrosine phosphatases (RPTPs) and nonreceptor protein tyrosine phosphatases (NRPTPs). Each of these two families of phosphatases can further be divided into subfamilies based on their distinctive structural features (Fig. 26-10).

### Protein tyrosine phosphatases are structurally different from serine–threonine phosphatases and contain a cysteine residue in their active sites

The catalytic domains of PTPs are different from those of the serine–threonine phosphatases, being approximately 200–300 amino acids in length and composed of a central  $\beta$  sheet enclosed by  $\alpha$ -helices. The highly conserved motif, (I/V)

HCXXGXGRS/TG, forms the base of the active-site cleft. The catalytic reaction mechanism is composed of two different steps. First, the nucleophilic thiolate anion of the active-site cysteine residue (Cys215 of PTP1B) attacks the phosphotyrosine on the substrate protein. A thiophosphate intermediate is then formed, accompanied by release of the dephosphorylated product. The second step involves hydrolysis of the thioester bond by an aspartate residue (Asp181 of PTP1B) to regenerate the free cysteine residue and phosphate. A commonly used PTP inhibitor, vanadate, reversibly inhibits PTPs by competing with phosphotyrosine for the active site. On the other hand, pervanadate irreversibly inhibits PTPs by binding to the active site and oxidizing the catalytic cysteine to cysteic acid.

The phosphotyrosine-recognition subdomain confers substrate specificity to PTPs by creating a deep pocket (9 Å) so that only the phosphotyrosine moiety is long enough to reach the cysteine nucleophile located at the base of this pocket; phosphoserine and phosphothreonine side chains are too short to be dephosphorylated. Dual-specificity kinases lack the phosphotyrosine-recognition subdomain and have a shallow catalytic-site cleft (6 Å) that is accessible to all three phosphorylated hydroxyamino acids. An example of a dual-specificity protein phosphatase is MAP kinase phosphatase (MKP)-1, which inactivates



**FIGURE 26-10** Schematic structures of nonreceptor protein tyrosine phosphatases (NRPTPs) and receptor protein tyrosine phosphatases (RPTPs). NRPTPs contain a catalytic domain and various regulatory domains. RPTPs are composed of an extracellular domain, a transmembrane domain and an intracellular domain with one or two catalytic domains. However, due to alternative splicing of some PTP genes, a single gene can produce both RPTP and NRPTP products from the same gene. Like RPTKs, the structural features of the extra-cellular domains divide the RPTPs into different families. For both RPTPs and NRPTPs, the non-catalytic domains contain motifs that serve a structural or regulatory function as well as help to define various subfamilies based on sequence similarity. Individual PTPs are designated by a name that is commonly used in the literature. Where necessary, the gene symbol is given in parentheses. (Adapted from Tonks. (2006))

MAPK by removing phosphate from both the threonine and tyrosine residues in the activation loop.

### Nonreceptor tyrosine phosphatases are cytoplasmic and have regulatory sequences flanking the catalytic domain

In addition to their specificity for phosphotyrosine, phosphotyrosine-specific NRPTPs appear to have selectivity for substrates (Tonks, 2006). For example, PTP1B prefers EGFR and INSR to many other phosphotyrosine-containing proteins. Such specificity is partly conveyed by a combination of intrinsic properties of the catalytic and regulatory domains that direct the enzyme to specific subcellular compartments where the preferred substrates are enriched. For PTP1B, its hydrophobic C-terminal tail directs this NRPTP to the cytoplasmic face of endoplasmic reticulum. Anchoring PTP1B at this strategic location may ensure that newly synthesized RPTKs are in their dephosphorylated quiescent state,

avoiding unwanted activation before arriving at their final destination in the plasma membrane. Other NRPTPs, SHP-1 and SHP-2, contain SH2 domains that direct the enzyme precisely to specific targets (Fig. 26-4). Despite considerable sequence homology, these two NRPTPs are thought to serve quite different functions. SHP-1 inactivates RPTKs by dephosphorylating autophosphorylation sites, while SHP-2 promotes downstream signaling cascades of RPTKs. SHP-2 interacts with the autophosphorylated PDGFR through its SH2 domain (Fig. 26-8). The activated PDGFR then phosphorylates SHP-2 and creates a binding site for Grb2, eventually resulting in activation of the MAPK pathway.

Regulatory domains that are specific for subcellular targets may have a second function in suppressing the catalytic activity of NPTPs during the quiescent state. Removal of the C-terminal motif on PTP1B responsible for target specificity is associated with an increase in catalytic activity. Similarly, association of the SH2 domain on SHP-2 with autophosphorylated RPTKs increases its activity by changing the conformation of the enzyme and allowing access of substrates to its active site.

## Receptor protein tyrosine phosphatases consist of an extracellular domain, a transmembrane domain and one or two intracellular catalytic domains

RPTPs can be divided into different classes by the structural features of the extracellular domain (Fig. 26-10), which includes the immunoglobulin-like, fibronectin III-like, MAM and carbonic anhydrase domains (Hunter, 1996). The immunoglobulin-like domains contain intramolecular disulfide bonds and a homophilic binding site for cell-cell adhesion molecules, such as neural cell adhesion molecule (NCAM). The fibronectin III-like domains originally were identified in the extracellular matrix protein fibronectin. They consist of conserved hydrophobic residues and may interact with integrins. The MAM domains are named because of their presence in meprins, A5 glycoprotein and PTPm. These domains contain four conserved cysteine residues. The carbonic anhydrase domains contain only one of the three histidine residues required for catalyzing the hydration of carbon dioxide and are unlikely to be catalytically active. It has been suggested that both the MAM and carbonic anhydrase domains play a role in cell adhesion (see Chapter 9).

The catalytic domains of RPTPs are in the intracellular region of the protein. Most RPTP families, except RPTP $\beta$ , contain two tandem catalytic domains. The proximal catalytic domain of most RPTPs contains all of the enzymatic activity. The distal catalytic domain appears to be inactive; in some cases, critical catalytic residues are missing. Despite the lack of enzyme activity, the distal catalytic domain may be important for mediating intra- or intermolecular interactions and biological activity of RPTPs. It has been shown that a chimeric CD45 in which the distal catalytic domain is replaced with an equivalent region from LAR becomes deficient in the induction of interleukin-2 secretion and ZAP-70 phosphorylation.

It was originally believed that PTP activity was constitutive and that tyrosine phosphorylation was regulated solely by activating the PTKs. However, it is now clear that PTPs play an active role in the regulation of tyrosine phosphorylation (Stoker, 2005; Tonks, 2006). This was suggested first by the discovery of RPTPs, such as CD45, that have a large extracellular domain reminiscent of that of RPTKs. Their activities are regulated by ligand binding to the extracellular domain. Chimeric studies fusing the intracellular domain of CD45 with the extracellular and transmembrane domains of the EGFR show that the CD45 intracellular catalytic domain is constitutively active. Addition of EGF suppresses the PTP activity of the chimera, suggesting that dimerization may negatively regulate RPTP activity (Desai et al., 1993). The mechanism of dimerization-induced inhibition has been revealed by crystallographic studies of an RPTP $\alpha$  fragment consisting of membrane-proximal region and the proximal catalytic domain (Tonks, 2006). These fragments form symmetrical dimers in which the active site of one molecule is blocked by an inhibitory wedge from the membrane-proximal region of the other. Based on this model, the inactive distal catalytic domain may promote tyrosine phosphatase activity of CD45 by competing with and inhibiting homodimerization of the proximal catalytic domain. In summary, activity of RPTPs may be diminished by ligand-induced dimerization, in contrast to activation by dimerization of RPTKs.

## Dual-specificity phosphatases are a diverse family defined by the signature cysteine-containing motif of PTPs

The functions and substrate specificity of DSPs are similarly diverse, but the active site can accommodate phosphoserine, phosphothreonine and other substrates as well as phosphotyrosine. As a result, some DSPs act to remove phosphates from serine/threonine or even from nonprotein substrates such as RNAs or even phosphatidylinositides like PTEN and the myotubularins (MTM) (Mruk & Cheng, 2010). Mutations in a myotubularin gene can produce a form of Charcot-Marie-Tooth peripheral neuropathy (Niemann et al., 2006). PTEN is a central player in the regulation of Akt and is thought to play roles in a wide range of activities, including neuronal differentiation, tumor suppression and brain disorders (Chang et al., 2007). Another well-characterized group of DSPs is responsible for inactivation of MAP kinases or cyclin-dependent kinases (DUSP) (see Ch. 25) (Figure 26-11).

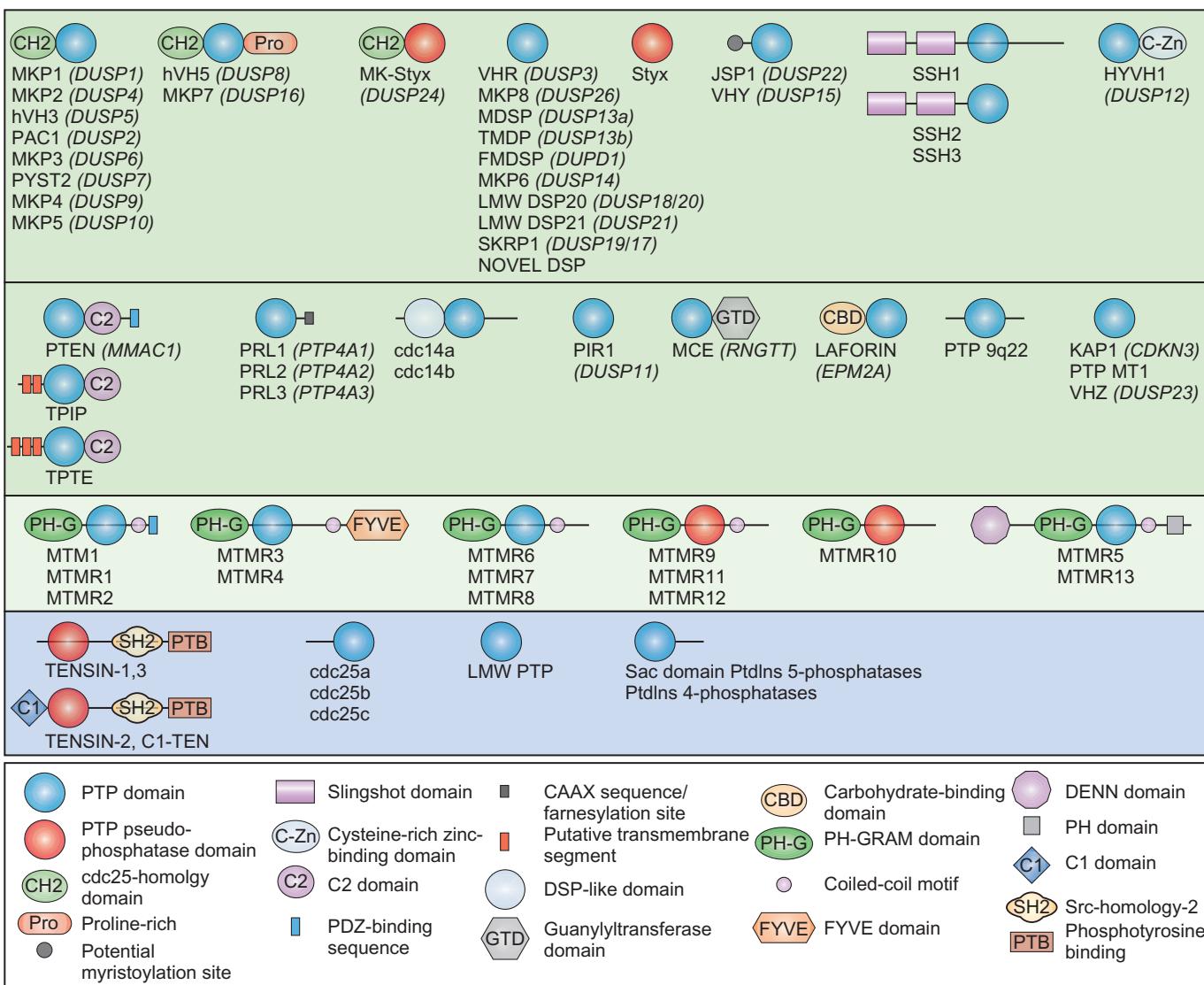
## ROLE OF TYROSINE PHOSPHORYLATION IN THE NERVOUS SYSTEM

Tyrosine phosphorylation plays a role in virtually every step in the development and functions of a neuron, including survival and differentiation, the extension of axons to their targets and synapse formation and function (Fig. 26-12) (see Chs 28, 29, and 32). Because of the plethora of effects of tyrosine phosphorylation on neuronal functions, the following is by no means a comprehensive review but focuses on several examples that demonstrate its significance for the nervous system (Fig. 26-12).

### Tyrosine phosphorylation is involved in every stage of neuronal development

Neuronal survival and differentiation are regulated by many factors including neurotrophins and the Trk family of RPTKs. The prototype neurotrophin, nerve growth factor (NGF), promotes the survival of sensory and sympathetic neurons during a period of programmed cell death in embryonic and early postnatal developmental stages as well as being required for survival of sensory neurons in adult. NGF is a target-derived neurotrophic factor that modulates the functions of the innervating axon terminals as well as gene expression in the distant cell body (Ch. 29).

Although NGF was discovered over half a century ago, its signal transduction mechanism (Fig. 26-9) remained elusive until the identification of a 140kDa protein, TrkA RPTK, as the NGFR (Reichardt, 2006). Studies in PC12 cells showed that NGF induces autophosphorylation of at least four distinct tyrosine residues on human TrkA. These are Y490 in the juxtamembrane region, Y674, Y675 in the catalytic domain and Y785 in the C-terminal tail. Each phosphotyrosine moiety serves a unique function in the NGF signaling cascade (Fig. 26-8). Y674 and Y675 are the first tyrosine residues phosphorylated and important for subsequent activation of intrinsic kinase activity. Phosphorylation of Y490 and Y785 creates SH2-binding



**FIGURE 26-11** Schematic structures of Dual Specificity Kinases, which have features of both PTPs and Serine/Threonine Phosphatases. They are classified as PTPs due to the presence of the signature motif HC(X)5R in the active site, but the catalytic domain is smaller than classical PTPs and they are more heterogeneous in structure. The non-catalytic domains contain motifs that serve a structural or regulatory function. These non-catalytic domains define various subfamilies based on sequence similarity, with individual DSPs designated by a name that is commonly used in the literature. Where necessary, the gene symbol is given in parentheses. Although the DSPs are related to PTPs and many have PTP activity, some remove phosphate from other moieties, such as RNA () and lipids (MTM, myotubularins). (Adapted with permission from (Tonks, 2006))

sites for SHC and PLC- $\gamma$ 1, respectively. In addition to SHC, phosphotyrosine 490 is also a binding site for Gab1 and FRS2 (or SNT1). Binding of Gab1 to and subsequent phosphorylation by TrkA recruits PI-3-kinase. Similarly, phosphorylation of FRS2 by TrkA plays a critical role in its subsequent interaction with the Grb2-Sos complex. In summary, association and proximity of signaling molecules to and/or their subsequent phosphorylation by TrkA appear to be an initial and essential part of signal transduction of NGF. The various members of the Trk family of RPTKs respond to different sets of neurotrophin molecules, including BDNF, Neurotrophin 3, NT-4 and/or NGF (Reichardt, 2006).

Activation of Trk receptors by neurotrophins may result in cellular proliferation, differentiation or survival. Exactly how different TrkA-induced signal transduction mechanisms are translated into each one of these functions is incompletely understood, but a number of signaling pathways may be affected directly or indirectly (Reichardt, 2006; Skaper, 2008). Proliferation and differentiation appear to be mediated by activation of the MAPK cascade, while survival is mediated by activation of PI-3-kinase and Akt. Signaling pathways activated by binding of neurotrophin to Trk receptors may also include phospholipase C $\gamma$ 1 and PKC, Akt, Ras/Raf and Erk, among others. (Chs 23 and 25).

## PLEIOTROPIC EFFECTS OF TRK RECEPTOR MUTATIONS

**Scott T. Brady**

Signaling through the RPTK family, Trk neurotrophin receptors activate multiple pathways and produce pleiotropic effects throughout the nervous system. In recent years, several human genetic diseases have been associated with loss-of-function mutations in specific Trk receptors (Reichardt, 2006). The phenotype is specific to the Trk receptor, based on the neurons that are normally dependent on a given Trk, as well as on the stage of development in which expression is critical and, potentially, on the genetic site of the mutation.

Several different types of mutation have been observed in the TrkA receptor gene, including nonsense, missense, frame shift and splice site mutations. The mutations are distributed throughout the various domains of the TrkA receptor. These loss-of-function mutations have been associated with a syndrome of congenital insensitivity to pain with anhidrosis (**CIPA**) (Indo, 2010). CIPA is a hereditary sensory and autonomic neuropathy, presenting as an autosomal recessive genetic disorder that is characterized by recurrent episodic fever, anhidrosis, absence of reaction to noxious stimuli, self-mutilating behavior, and mental retardation. Loss of pain sensitivity is particularly pernicious, because serious injuries can occur without being detected. Phenotypes associated with CIPA result in large part from embryonic loss of NGF-dependent neurons in the PNS, including nociceptive sensory and sympathetic neurons, and in the CNS, including cholinergic neurons in the forebrain.

Mutations in the TrkB receptor gene present a very different phenotype. TrkB null mice die shortly after birth, whereas mice with haploinsufficiency survive and exhibit a distinctive set of phenotypes (Chao, 2003). These include expected phenotypes such as impaired LTP and loss of specific neuronal populations, but also less obvious changes. These include elevated striatal dopamine, loss of mechanosensitivity, and obesity. These mouse

phenotypes are consistent with human disease. For example, a dominant mutation in TrkB (Y722C) that impairs TrkB kinase signaling has been described in a patient with severe hyperphagic obesity and severe impairments in nociception, learning and memory (Yeo et al., 2004). Interestingly, point mutations in specific signaling motifs of TrkB can lead to different phenotypes. Thus, the Y816F mutation abolishes signaling through the PLC $\gamma$ 1 pathway (Ch. 25), leading to reductions in synaptic plasticity, but not affecting neuronal survival or the set of transcription responses that are mediated by the Ras/MAPK and PI3K pathways, because these are activated by a different domain (Minichiello, 2009) (see Figure 26-9A). Knowledge of the molecular architecture of the Trk receptors and delineation of distinct signaling modalities provide a molecular basis for understanding the phenotypes of mutations in mouse and human.

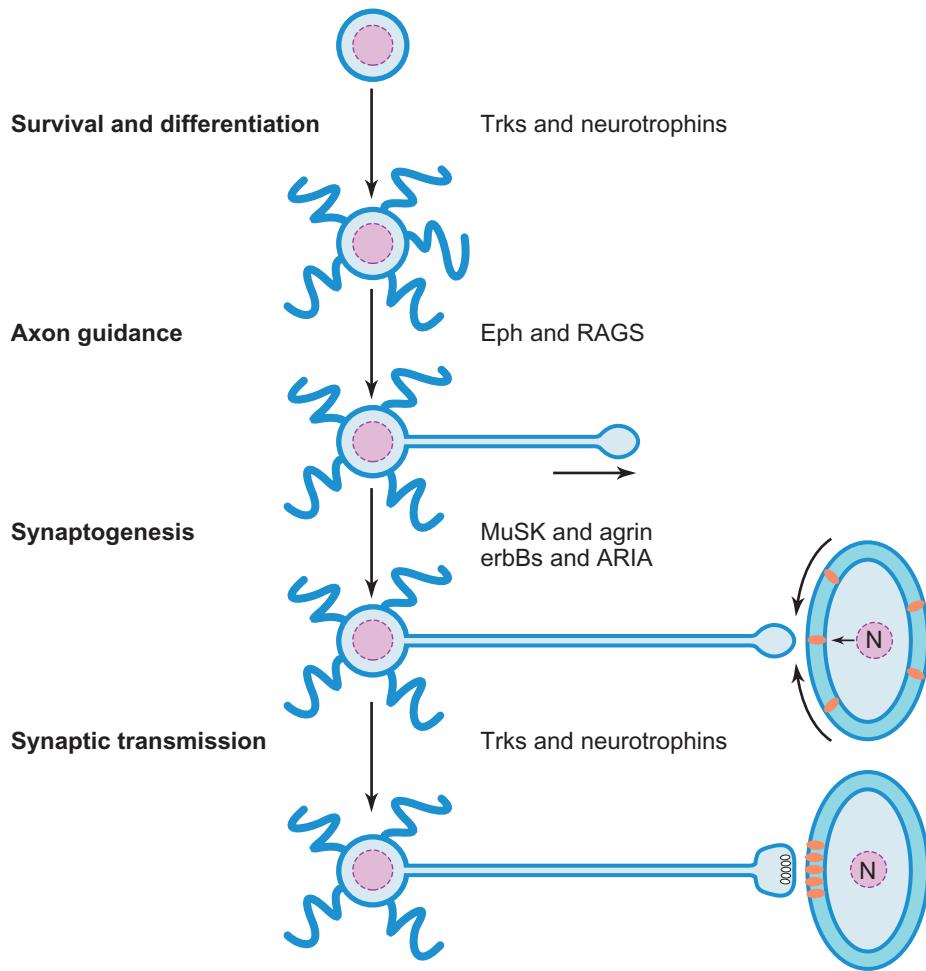
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During development, axons often need to navigate through long distances before finding their targets. One of the best-studied model systems of axon guidance in vertebrates is perhaps the retinotectal system (McLaughlin & O’Leary, 2005). Anterior and posterior retinal axons project to the posterior and anterior tectum, respectively. Similarly, the dorsal and ventral retinal axons innervate the ventral and dorsal tectum respectively. This reversal of projection is important for the rectification of the upside-down and back-to-front retinal image. Repulsive axon guidance signals (RAGS) were identified as an important axon guidance molecule in this system and RAGS was found to be a ligand for a member of the Eph RPTK family (Drescher et al., 1995). Ligands for Eph family RPTK or ephrins are either glycoprophosphatidylinositol (GPI)-anchored or transmembrane proteins. Their biological activity is lost if they are detached from the membrane. This implies that Eph-mediated axon guidance is limited to short-range contact-dependent activity. Accordingly, the Eph family of receptors can be classified into those (EphA) interacting with GPI-linked ligands

(ephrin-A), and those (EphB) with transmembrane ligands (ephrin-B). Signaling with the Eph pathway is bidirectional with the EphR having RPTK activity and the ligands activating Src family NRPTK and small GTPases. EphR/ephrins are now understood to be important for axon growth and guidance in regeneration as well as during development and play a role in proliferation of astrocytes following injury (Goldshmit et al., 2006). Members of the EphR family play important roles not only in axon guidance, but in regulating the actin cytoskeleton, cell adhesion and cell migration in both health and disease states (Pasquale, 2008).

Steering of an axon in different directions towards its destination is largely mediated by changes in the cytoskeleton in a structure at the leading edge of an axon known as the growth cone (Chs 6 and 32). The growth cone consists of finger-like protrusions known as filopodia interspersed by web-like structures called lamellipodia. Filopodia and lamellipodia are highly dynamic structures. Attractive extracellular cues favor extension of filopodia by stabilizing and



**FIGURE 26-12** Examples of the role of protein tyrosine kinases and protein tyrosine phosphatases in the lifetime of a neuron. (N). Tyrosine phosphorylation appears to be an important signal-transduction mechanism in every step in the development of a neuron, starting from the survival and differentiation of stem cells, to axon guidance and synaptogenesis and to synaptic transmission at a mature synapse. Examples of each step are given on the right, indicating the receptors and their ligands. *Eph*, erythropoietin-producing hepatocellular factor; *RAGS*, repulsive axon guidance signal; *MuSK*, muscle-specific kinase; *ARIA*, acetylcholine-receptor-inducing activity.

extending the underlying actin filaments while a repulsive cue retracts filopodia by collapsing the actin filaments. Mechanisms converting signals from extracellular cues into changes in intracellular cytoskeletal dynamics in the growth cone are gradually emerging. These mechanisms include various PTKs, PTPs and their substrates. The extracellular cues for axon guidance are ephrins, semaphorins, Slits and netrins (Dickson, 2002). Receptors for ephrins (Eph) and some semaphorins (e.g. Met for sema4D) are RPTKs. The Slit receptor family members (Robo) are not RPTKs but are associated with a NRPTK (Abl) and its substrate (Ena), both involved in Robo signaling. Furthermore, Robo itself carries a conserved tyrosine residue, dephosphorylation of which by a RPTP has been suggested to be a prerequisite for Robo to transmit the repulsive signal from Slit.

What is the molecular mechanism that links changes in tyrosine phosphorylation of surface receptors to alterations in the cytoskeletal dynamics in the growth cone? Multiple signaling

pathways have been reported downstream of EphR including Src and Fyn NRPTK, Ras/MAPK, and Rho/Rac small GTPases (Goldshmit et al., 2006). One of these molecules is Nck, an adaptor molecule consisting of both SH2 and SH3 domains. The SH2 domain of Nck interacts with a phosphotyrosine moiety on activated Eph RPTKs while its SH3 domains recruit PAK and WASP. Since both PAK and WASP regulate reorganization of cytoskeleton, Nck may act as an intermediary between EphR activation and growth cone navigation. Interestingly, the ephrin-EphR interaction constitutes a bidirectional communication, i.e., association of this ligand-receptor pair not only activates the Eph RPTK but also induces tyrosine phosphorylation of the cytoplasmic domain of ephrin. A close homolog of Nck, Grb4 binds to the phosphotyrosine residue on the cytoplasmic tail of ephrin and results in actin depolymerization. Similar to Nck, Grb4 interacts with a number of modulators of cytoskeletal dynamics, including PAK1, Abl-interacting protein-1 (Abi-1) and Cbl-associated protein (CAP).

## Tyrosine phosphorylation has a role in the formation of the neuromuscular synapse

The acetylcholine receptor (AChR) is concentrated at the postsynaptic membrane of the neuromuscular junction at a density of 10,000 receptors/ $\mu\text{m}^2$ , about three orders of magnitude greater than extrasynaptic membrane (Wu et al., 2010) (see Chs 13 and 44). The high concentration of AChRs at the neuromuscular junction allows rapid, reliable and efficient synaptic transmission. Two mechanisms contribute to the enrichment of AChRs at the neuromuscular junction: (1) clustering of pre-existing, diffusely distributed surface AChRs and (2) local synthesis of receptors by subsynaptic nuclei. Both mechanisms are mediated by tyrosine phosphorylation.

Clustering of preexisting AChRs on the postsynaptic membrane is triggered by a motor-neuron-derived extracellular matrix protein called agrin, from the Greek word *ageirein* meaning ‘to assemble’ (McMahan, 1990). The molar ratio of agrin to AChRs at the neuromuscular junction is between 1:50 and 1:100, suggesting that agrin induces AChR clustering through some intracellular signal transduction mechanisms rather than via any structural constraints. Tyrosine phosphorylation of the AChR has been suggested to play a role in its clustering at the neuromuscular junction. First, agrin induces tyrosine phosphorylation of the AChR prior to clustering of the receptor in muscle. All agents that induce AChR clustering in muscle cells in culture also induce tyrosine phosphorylation of the AChR. These agents include agrin, rapsyn, electrical fields and latex beads. In contrast, PTK inhibitors that inhibit AChR tyrosine phosphorylation also inhibit AChR clustering.

The receptor for agrin at the neuromuscular junction is an RPTK known as muscle-specific kinase (MuSK). The extracellular domain of MuSK resembles that of the ROR family of RPTKs, while the kinase domain is similar to that of the Trk neurotrophic receptor (Fig. 26-3). MuSK is expressed at low concentrations in proliferating myoblasts and is induced upon differentiation and fusion. It is downregulated dramatically in mature muscle except at the neuromuscular junction. These properties are consistent with a role of MuSK in muscle development and in the functioning of neuromuscular junctions, which is mediated by agrin. However, MuSK and agrin do not interact directly but require a member of the low-density lipoprotein receptor family, Lrp4, that associates with MuSK through its extracellular domain (Wu et al., 2010). This MuSK/Lrp4 complex along with a variety of associated proteins including NRPTKs (Abl and Src), other signaling molecules (Rho, Pak1) and adapter proteins act to organize many components of the neuromuscular junction, including AChR and other proteins.

Among the other proteins recruited by agrin to the neuromuscular junction is the ErbB family of RPTKs. This family contains four known members: ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4. The last three members are expressed in developing muscle and cultured myotubes. Although both AChRs and ErbBs are recruited to the neuromuscular junction by agrin, ErbBs are localized in the troughs of the junctional folds while AChR sits on the tip, suggesting distinct downstream targeting mechanisms. ErbBs at the neuromuscular junction are activated by another motor-neuron-derived factor known as neuregulin. Their activation is responsible for localized transcription

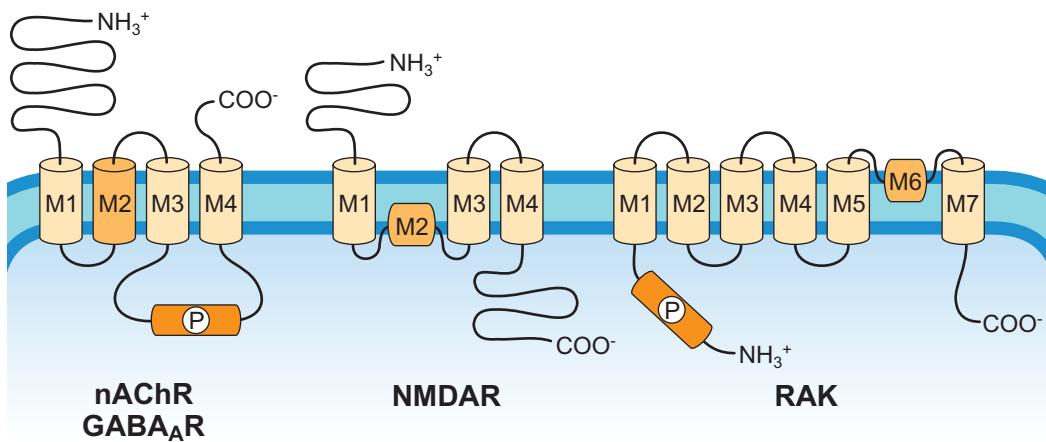
of AChR in subsynaptic nuclei. Neuregulin-activated ErbBs are autophosphorylated; the induced phosphotyrosine moiety acts as anchor for an adaptor molecule—SHC—leading to activation of the MAPK pathway and subsequent increased transcription of the AChR gene. In accordance with the significance of tyrosine phosphorylation in this signaling pathway, a PTP, SHP2, has been shown to be a negative regulator.

## Tyrosine phosphorylation contributes to the formation of synapses in the central nervous system

The action of neurotrophins and their corresponding Trk RPTKs play important roles in formation and regulation of synapses in the brain (Hennigan et al., 2007; Reichardt, 2006) (Ch. 29). Neurotrophins affect formation of both excitatory and inhibitory synapses through presynaptic as well as postsynaptic mechanisms. In excitatory synapse formation, addition of exogenous BDNF and NT3 significantly increases the amount of docked synaptic vesicles in primary cultured hippocampal neurons. Similarly, transgenic mice overexpressing BDNF experience an increase in synaptic density (Causing et al., 1997) whereas those deficient in BDNF, TrkB (preferred receptor for BDNF) or TrkC (preferred receptor for NT3) display the opposite (Martinez et al., 1998). Similar studies have reported potentiation of inhibitory synapse formation by BDNF/TrkB.

Tyrosine phosphorylation plays an important role in synaptic transmission and plasticity. The significance of tyrosine phosphorylation in synaptic plasticity was perhaps first revealed by the impairment of long-term potentiation (LTP) by tyrosine kinase inhibitors. Induction of long-term depression (LTD) has subsequently been reported to be susceptible to these inhibitors as well. As the role of neurotrophins and Trk receptors on synaptic function became recognized (see Ch. 29), the effects of these inhibitors were thought to act through these RPTK. For example, mutant mice lacking BDNF or TrkB exhibit diminished LTP in the CA1 region of the hippocampus (Minichiello et al., 1999). In particular, the role of TrkB and downstream signaling pathways for LTP and learning is now well documented (Minichiello, 2009). However, the importance of tyrosine phosphorylation on synaptic function is not restricted to Trks and neurotrophins. Genetic knock-out experiments have identified a role for NRPTKs in these processes as well. For example, mutant mice deficient in Fyn exhibit diminished LTP in the hippocampus (Grant et al., 1992). Reintroduction of Fyn to the adult brain in Fyn knock-out mice restores LTP, suggesting that Fyn plays a direct role in the manifestation of LTP independent of its developmental effects (Kojima et al., 1997). Conversely, transgenic mice over-expressing Fyn display a lower threshold for the induction of LTP (Lu et al., 1999). Fyn and Src activity may be downstream of the Trk signaling pathway (Minichiello, 2009) or may be regulated through some other aspect of synaptic activity.

The mechanisms by which BDNF/TrkB enhances the induction of LTP are complex and include both pre- and postsynaptic mechanisms (Fig. 26-9). Furthermore, some actions of the TrkB receptor are local in the synapse, while others require retrograde signaling through retrograde transport of signaling



**FIGURE 26-13** Transmembrane topology of three ligand-gated ion channel subunits and their potential domains for tyrosine phosphorylation. Both the acetylcholine receptor (*AchR*) and GABA<sub>A</sub> receptor (*GABA<sub>A</sub>R*) subunits have four transmembrane domains with both the N- and C-termini on the outside. Tyrosine phosphorylation sites are present within the large intracellular loop between the third and fourth transmembrane domains. *N*-methyl-d-aspartate (*NMDA*) receptor subunits have a somewhat different topology. They have three transmembrane domains and a membrane-spanning domain. The delayed rectifier potassium channel (*RAK*) has six transmembrane domains and a membrane-spanning domain. Mutagenesis studies have revealed the intracellular N-terminus as the tyrosine-phosphorylation site-bearing region, although other studies have suggested the presence of other tyrosine-phosphorylation sites.

endosomes (Cosker et al., 2008). Thus, BDNF elevates pre-synaptic cytosolic calcium level and increases vesicular neurotransmitter release, but postsynaptic TrkB activation leads to phosphorylation of glutamate receptors and opening of TRP channels through phospholipase C- $\gamma$ 1 (Minichiello, 2009; Reichardt, 2006) (Figure 26-9B).

Neurotransmitter receptors and voltage-gated ion channels play a pivotal role in modulating synaptic efficacy and plasticity. Many of these receptors and ion channels in both the peripheral and central nervous systems are tyrosine phosphorylated (Davis et al., 2001). Tyrosine phosphorylation of these surface signal-transducing molecules significantly modulates their electrophysiological properties, producing a prominent effect on synaptic transmission and/or plasticity.

### Acetylcholine Receptors

The AChR in the neuromuscular junction is one of the best characterized neurotransmitter receptors (Ch. 13). Upon binding acetylcholine, this ligand-gated ion channel depolarizes the sarcolemma and triggers muscle contraction, so it is important to regulate AChR activity and location. The AChR is composed of five subunits,  $\alpha_2\beta\gamma\delta$  (embryonic) or  $\alpha_2\beta\epsilon\delta$  (adult). Each subunit has four transmembrane domains; both the N- and C-termini are in the extracellular space. Between the third and fourth transmembrane domains is a large cytoplasmic region containing a single conserved tyrosine residue in the cytoplasmic loop of each of the  $\beta$ ,  $\gamma$  and  $\delta$  subunits (Fig. 26-13). The AChR is highly tyrosine-phosphorylated in adult muscle, in contrast to its low tyrosine phosphorylation in cultured rat myotubes,  $<0.001\mu\text{mol phosphate/mol subunit}$ . Tyrosine phosphorylation of the AChR increases as muscle is innervated during development or in culture. In contrast, denervation results in tyrosine

dephosphorylation of the AChR. Agrin-mediated activation of the RPTK MuSK is required for tyrosine phosphorylation of the AChR, leading to clustering and anchoring of AChR at the neuromuscular junction (Wu et al., 2010). This modification is likely due to activation of Src family NRPTKs by MuSK and appears to stabilize the AChR clusters rather than being necessary to create the cluster (Wu et al., 2010). In addition, tyrosine phosphorylation of the AChR affects the rate of receptor desensitization (Hopfield et al., 1988) and may thus regulate receptor sensitivity at the neuromuscular junction.

### N-Methyl-d-Aspartate Receptors

Glutamate is the major excitatory neurotransmitter in the central nervous system (Ch. 17). Its receptors can be divided into three types: AMPA/kainate, NMDA and metabotropic receptors. NMDA receptors are composed of two different types of subunit: NR1 and NR2. They play an important role in the induction of synaptic plasticity and excitotoxicity. The activity of NMDA receptors can be enhanced by an NRPTK-Src (Ali & Salter, 2001). Application of Src or an Src activating peptide potentiates NMDA receptor activity in cells and inside-out membrane patches. Similar potentiation of NMDA receptor activity can be achieved by other Src family NRPTKs, such as Fyn. Increased NMDA-evoked channel activity is attributed to an increase in probability and duration of channel opening and a decrease in duration of channel closing. Cumulative evidence has suggested that tyrosine phosphorylation of the NR2 subunits regulates NMDA receptor function. For instance, the NR2A and 2B but not NR1 subunits are tyrosine-phosphorylated *in vivo* (Lau & Huganir, 1995; MacDonald et al., 2006). Both NR2A and NR2B can be phosphorylated by Src *in vitro*. Heterologous cells expressing NR1 and NR2A subunits exhibit

increased NMDA-induced current in the presence of v-Src, while cells expressing NR1 and other NR2 subunits display no change in NMDA-evoked current. Finally, mutation of tyrosine residues in the cytoplasmic domain of NR2A to phenylalanine abolishes Src-induced NMDA receptor activity.

Both NMDA receptor and Fyn have been implicated in the induction of LTP. Since Src family tyrosine kinases are known to tyrosine phosphorylate and activate NMDA receptor, it is enticing to speculate that Src family kinases and NMDA receptors somehow act in a concerted fashion in the expression of LTP. Other PTKs may affect NMDA function. For example, the axon guidance receptor EphB2 is expressed in adult hippocampus and interacts with the NR1 subunit of the NMDA receptor. Activation of EphB2 by ephrin-B2 has also been shown to induce tyrosine phosphorylation of the NR2B subunit with an accompanying increase in NMDA-receptor-mediated calcium ion influx (Takasu et al., 2002).

The regulation of NMDA receptors by Src-family kinases is enhanced by the presence of scaffold proteins in the postsynaptic domain. Src-induced increases in NR1-NR2A receptor activity are promoted by coexpression of the postsynaptic density protein known as PSD-95 (Tezuka et al., 1999). PSD-95 is a scaffolding protein consisting of multiple protein-protein interaction domains—three N-terminal PDZ domains, an SH3 domain and a C-terminal guanylate kinase domain. The first two PDZ domains interact with the NR2 C-terminal tails, while the third PDZ domain binds the SH2 domain of Fyn. In this fashion, PSD-95 serves as an adaptor, bringing Fyn and its substrate in proximity to each other.

### GABA Receptors

GABA is one of the major inhibitory neurotransmitters in the central nervous system (see Ch. 18) and exhibits multiple modes of regulation by tyrosine phosphorylation. The subunits of the GABA<sub>A</sub> receptor (GABA<sub>A</sub> R) consist of  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$  and  $\pi$ ; subunits found in the brain are mainly from the first three classes. Similar to AChR, the GABA<sub>A</sub> R complex is believed to be heteropentameric, with four transmembrane domains in each subunit. The intracellular region between the third and fourth transmembrane domains contains a number of consensus protein phosphorylation sites (Fig. 26-12). Coexpression of GABA<sub>A</sub> R subunits  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2L$  with v-Src induces tyrosine phosphorylation on both  $\beta$  and  $\gamma$  subunits, with a concomitant increase in GABA-mediated whole-cell current (Moss et al., 1995). Mutation of two tyrosine residues (Y365/367) on the cytoplasmic domain of the  $\gamma$  subunit abolishes not only the induction of tyrosine phosphorylation but also functional effects of v-Src, suggesting that direct tyrosine phosphorylation of GABA<sub>A</sub> R increases its activity. Although direct tyrosine phosphorylation of the GABA<sub>A</sub> R augments channel activity, activation of some RPTKs may indirectly inhibit GABA<sub>A</sub> AR function. For instance, activation of PDGFR inhibits GABA<sub>A</sub> R in hippocampal neurons as a consequence of activating PLC- $\gamma 1$ , generating IP<sub>3</sub> and elevating intracellular calcium. In addition to direct enhancement of channel activity, PTKs can indirectly increase GABA-evoked inhibitory current by recruiting intracellular GABA<sub>A</sub> R to the surface of postsynaptic membrane. In central neurons, insulin rapidly increases expression of functional postsynaptic GABA<sub>A</sub> R in a tyrosine kinase-dependent

manner, increasing the amplitude of miniature inhibitory postsynaptic currents.

### Voltage-Gated Ion Channels

Many voltage-gated ion channels also appear to be regulated by tyrosine phosphorylation (Reichardt, 2006). These include voltage-gated calcium, potassium and sodium ion channels whose function can either be enhanced or impeded by tyrosine phosphorylation. For example, voltage-gated L-type calcium ion channel activity is augmented by tyrosine phosphorylation. Voltage-gated calcium ion channels are composed of  $\alpha_1$ ,  $\alpha_2\delta$  and  $\beta$  subunits. The  $\alpha_1$  subunit forms the pore of the calcium ion-selective channel. It consists of six transmembrane domains with both the N- and C-termini in the cytoplasmic side, carrying a number of potential phosphorylation sites. It is believed that both cytoplasmic ends of the channel impose an inhibitory tone on functional activity; upregulation of channel activity by protein phosphorylation may be attributed to the relief of this inhibition. The L-type calcium ion channel is tyrosine-phosphorylated and activated in cerebellar granule neurons in response to stimulation by insulin-like growth factor-1 (IGF-1) (Bence-Hanulec et al., 2000). This potentiation induced by IGF-1 appears to be mediated by phosphorylation of Y2122 on the  $\alpha 1C$  subunit via the action of Src.

In contrast, a delayed rectifier potassium ion channel, Kv1.2, provides an example of downregulation by tyrosine phosphorylation. Kv1.2 coexpressed with the M1 muscarinic AChR in *Xenopus* oocytes is inhibited by carbachol, a nonmetabolizable analog of acetylcholine. Activation of the M1 muscarinic receptor releases diacylglycerol and IP<sub>3</sub> from the hydrolysis of PIP<sub>2</sub>. Diacylglycerol activates PKC and IP<sub>3</sub> releases calcium from intracellular stores. Possibly by their action on a PYK2-related PTK, calcium and PKC eventually lead to tyrosine phosphorylation of Kv1.2 on Y132 in the cytoplasmic domain near the N-terminus, resulting in an impaired functional response. Tyrosine phosphorylation of Kv1.2 and its concomitant reduction in channel activity can be reversed by RPTP $\alpha$ . Similarly, a related potassium channel, Kv1.5, is tyrosine-phosphorylated and suppressed when co-expressed with v-Src in transfected cells. *In vivo* association between hKv1.5 and Src is mediated by proline-rich sequences on the potassium ion channel and Src SH3 domain.

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