‘Eph’ective signaling: forward, reverse and crosstalk

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Summary

The Eph receptors comprise the largest group of receptor tyrosine kinases and are found in a wide variety of cell types in developing and mature tissues. Their ligands are the ephrins, a family of membrane-bound proteins found in lipid rafts. In the past decade, Eph receptors and ephrins have been implicated in a vast array of cellular processes. Unlike other receptor tyrosine kinases, however, the Eph receptors seem to be geared towards regulating cell shape and movement rather than proliferation. Studies have uncovered intricate signaling networks that center around the ligand-receptor complex, and this may account for the broad repertoire of functions of Eph proteins. Deciphering the bi-directional pathways emanating from an Eph receptor-ephrin complex will not only help us to understand basic biological processes, but may also provide important insight into disease.

Key words: Receptor tyrosine kinase, Growth cone, Axon guidance, Synapse, Angiogenesis, Rho, Ras

Introduction

In 1987, a receptor was identified in a search for tyrosine kinases involved in cancer (Hirai et al., 1987). The new receptor was named Eph, after the erythropoietin-producing hepatocellular carcinoma cell line from which its cDNA was isolated. Today, 16 receptors (EphA1-10 and EphB1-6) and 9 ligands are known in vertebrates (14 receptors and 8 ligands in mammals; http://cbweb.med.harvard.edu/eph-nomenclature) (Manning et al., 2002), and hundreds of articles have described their structure, localization and function. What may have driven the remarkable expansion of the Eph and ephrin families during evolution? Eph gene duplication may have served to introduce subtle functional differences between Eph receptors or ephrins, as well as establish a combinatorial code of expression patterns that regulate complex tissue architecture. An expanded exploration of the evolution of Eph genes can be found in the literature (Drescher, 2002), as well as reviews on a variety of different aspects of Eph function (Holmberg and Friesen, 2002; Kullander and Klein, 2002; Wilkinson, 2001). Here we highlight recent findings addressing the role of Eph receptors and ephrins, emphasising the bi-directional signals that regulate cell morphology and motility.

Regulation of Eph expression

The highly dynamic and complex spatiotemporal expression patterns of the Eph receptors and ephrins are controlled by a variety of transcription factors, including many homeobox genes (Barbieri et al., 2002; Koshiha-Takeuchi et al., 2000; Logan et al., 1996; Mui et al., 2002; Schulte and Cepko, 2000; Stadler et al., 2001; Theil et al., 1998; Yuasa et al., 1996). Remarkably, the transcription factor Tcf-4, which is activated by nuclear β-catenin, can simultaneously upregulate certain Eph receptors and downregulate their ligands (Batlle et al., 2002). In the intestinal epithelium this establishes patterns of EphB and ephrin-B expression that demarcate proliferative versus differentiated cells, respectively, and determine the position of cells along the crypt-villus axis. Interestingly, cadherin-mediated cell-cell adhesion and the Notch/Delta pathway can also regulate Eph expression (De Bellard et al., 2002; Orsulic and Kemler, 2000). More recently, the translation of Eph receptor mRNA has also been found to be modulated. Restricted translation may allocate Eph proteins to distinct subcellular compartments for localized functions in processes such as axon guidance (Brittis et al., 2002). Thus, precise transcriptional and translational regulation could determine the combinatorial patterns of Eph receptors and ephrins.

Structural features

Despite their large number, the Eph receptors have a remarkably conserved domain structure, which is shown in Fig. 1. The EphA receptors, which are more similar to each other than to the EphB receptors, preferentially bind the glycosylphosphatidylinositol (GPI)-linked ephrin-A ligands. The EphB receptors, by contrast, bind the transmembrane ephrin-B ligands. Recent structural characterization of Eph proteins has provided important insight into their functional properties (Himanen and Nikolov, 2003a; Himanen and Nikolov, 2003b). X-Ray crystallography of a ligand-receptor complex depicts a high affinity interaction that is mediated by an ephrin loop inserted into an Eph receptor cleft (Fig. 1C). Interestingly, peptides mimicking the ephrin loop can inhibit this Eph receptor-ephrin high-affinity interaction (Koolpe et al., 2002) and can discriminate between individual Eph receptors. By contrast, the natural ephrin ligands show promiscuity within a receptor subclass. The high affinity Eph receptor-ephrin interface is accompanied by a distinct and
lower affinity interface that probably mediates the assembly of tetrameric complexes (Fig. 1C).

Recent studies show that engagement with an ephrin induces a conformational change in the cytoplasmic portion of the Eph receptor (Fig. 1B) (Wybenga-Groot et al., 2001). This is triggered by phosphorylation of two juxtamembrane tyrosine residues, which relieves the inhibition of the juxtamembrane segment on the kinase domain (Zisch et al., 2000). This phosphorylation also establishes binding sites for the SH2 domains of several proteins. Occupancy of the juxtamembrane binding sites and additional phosphorylation may further stabilize the active conformation of the Eph receptor. The transmembrane ephrin-B ligands also become phosphorylated on conserved cytoplasmic tyrosines upon binding to an EphB receptor (Bruckner et al., 1997; Holland et al., 1996; Kalo et al., 2001). This tyrosine phosphorylation may induce a conformational change in the hairpin structure in the C-terminal half of the cytoplasmic domain and allow binding of SH2 domains (Song et al., 2002).

**Functional versatility of Eph proteins**
The Eph receptors and ephrins are among the molecular workhorses that trigger cellular responses to cues in the environment in both developing and mature structures. One prominent responsibility of the Eph proteins is to establish cell positioning and maintain cellular organization. In many developing regions of the central nervous system, Eph receptors and ephrins show complementary patterns of expression. This suggests that they function in the initial stages of cellular compartmentalization in areas such as the hindbrain (Pasini and Wilkinson, 2002). Eph bi-directional signals are important for inhibiting cell intermingling between adjacent rhombomeres in Zebrafish (Mellitzer et al., 1999; Xu et al., 1999), and similar mechanisms probably determine the segmental identity of the somites (Durbin et al., 1998) and the regional migration of neural crest cells (Krrl et al., 1997; Smith et al., 1997; Wang et al., 1997). Surprisingly, ephrin-B ligands can perform a dual function during neural crest cell migration. They repel ventrally migrating neural crest cells away from a dorsolateral migratory path and promote the later migration of melanoblast neural crest cells to the skin along the dorsolateral pathway (Santiago and Erickson, 2002). These differential functions probably exemplify the complexity of cell-type-specific Eph signaling, which can result in cell repulsion or cell adhesion and attraction. Eph receptors and ephrins also define parasagittal stripes of Purkinje cells in the developing cerebellum (Karam et al., 2000). Thus, Eph proteins help determine the initial sorting and positioning of cells in a wide spectrum of neural tissues. The function of Eph receptors and ephrins also extends to the processes governing axon guidance and a discussion of this.
Recent advances also suggest that Eph receptors and ephrins regulate the development and function of neuromuscular junctions (Lai et al., 2001) and central synapses (Gerlai, 2001). During synaptogenesis, the Eph receptors help establish (Dalva et al., 2000) and modify the postsynaptic specialization (Ethell et al., 2001) by transmitting signals to the actin cytoskeleton through the Rho-family of small GTPases (Irie and Yamaguchi, 2002; Penzes et al., 2003). EphB receptors also modulate NMDA-receptor-mediated calcium influx via a Src-family kinase pathway (Takasu et al., 2002) and could have a direct impact on synaptic transmission (Henderson et al., 2001; Contractor et al., 2002). Intriguingly, EphA/ephrin-A signals mediate a form of crosstalk between glial cells and neurons, which regulates the morphology of excitatory synapses in the mature hippocampus (Murai et al., 2003). Thus, both EphA and EphB receptors can regulate the structural and functional properties of synapses and probably participate in cognitive processes including learning and memory formation (Murai and Pasquale, 2002).

Eph proteins also play a critical role in the cellular organization and function of non-neural tissues. In the cardiovascular system, ephrin-B2 and EphB4 are preferentially expressed on arterial and venous endothelium, respectively (Wang et al., 1998). The similar phenotypes of EphB4+ and ephrin-B2-knockout mice suggest that reciprocal signaling is important for vascular development and remodeling in the embryo (Adams et al., 1999; Gerety et al., 1999). EphB2, EphB3 and ephrin-B1 have also been implicated in embryonic vascularization and their localization suggests that signaling occurs not only between the arterial and venous compartments but also between endothelial cells and the surrounding mesenchyme (Adams et al., 1999). Intriguingly, EphB proteins may contribute to the organization of the vascular network by mediating neuro-arterial interactions (Mukouyama et al., 2002). These findings have important implications for understanding and treating cancer because Eph proteins probably regulate angiogenic processes associated with tumor growth (Brantley et al., 2002; Dodelet and Pasquale, 2000; Ogawa et al., 2000; Pandey et al., 1995). Interestingly, EphB4 and ephrin-B2 also play a role in erythropoiesis by influencing hematopoietic cell lineages that share a common ancestry with endothelial cells (Suenobu et al., 2002; Wang et al., 2002b). Furthermore, Eph proteins play a role in platelet clustering and hence may be important for blood clotting at sites of vascular injury (Prevost et al., 2002).

In summary, the Eph receptors and ephrins display extensive functional versatility. Their activities result from bi-directional signals propagated downstream of the ligand-receptor complex. In the next sections we summarize some of the most recent findings on Eph receptor forward signaling, ephrin-mediated reverse signaling, and crosstalk with other receptors.

**Fig. 2.** Forward and reverse signals communicated downstream of the Eph-ephrin complex. Some of the known EphA–ephrin-A or EphB–ephrin-B signaling pathways are highlighted. Note that SH2 and PDZ refer to a number of identified proteins containing SH2 or PDZ domains. Grb4 is the only SH2-domain-containing protein known to bind to ephrins.
Forward signaling
How can the multi-functional properties of the Eph receptors be reconciled? One way is through differential kinase-mediated forward signaling. Recent advances have shed light upon Eph signaling mechanisms that regulate cell morphology, adhesion and migration (Fig. 2).

Eph signaling through Rho family GTPases
Increasing evidence indicates that the Eph receptors regulate actin dynamics through small GTPases of the Rho family (Rho, Rac and Cdc42). Rho GTPases cycle between an active, GTP-bound conformation and an inactive GDP-bound conformation. They control cell shape and movement by promoting the formation of stress fibers (Rho), lamellipodia (Rac) and filopodia (Cdc42) (Nobes and Hall, 1995). In neurons, Rho activation inhibits neurite outgrowth and promotes growth cone collapse and axon retraction (Dickson, 2001; Luo, 2000; Yuan et al., 2003). Rac and Cdc42 play an antagonistic role to Rho in neuronal morphology by enhancing the formation of the F-actin meshwork in growth cone lamellipodia (Rac) and promoting the extension of filopodia (Cdc42) (Kozma et al., 1997; Yuan et al., 2003).

EphA receptors can directly activate Rho GTPases through the recently identified exchange factor Ephexin (Shamah et al., 2001). Exchange factors activate GTPases by catalyzing the replacement of GDP with GTP. Ephexin is preferentially expressed in the nervous system and constitutively binds the kinase domain of EphA receptors. Interestingly, ephrin-A1 treatment of cultured neurons potentiates Ephexin-mediated exchange on Rho. The activation of Rho and its downstream effectors, Rho-associated kinases (RhoA), promotes ephrin-A-induced signals to initiate growth cone collapse (Wahl et al., 2000). In 293T and melanoma cells ephrin-A-induced Rho activation causes the retraction of cell processes, cell rounding/detachment, and membrane blebbing, and this appears to depend on the adaptor protein Crk (Lawrenson et al., 2002). Rho activity may mediate these events by stabilizing actin filaments and promoting actomyosin contractility (Dickson, 2001; Luo, 2000). Recent data show that actin filament stabilization alone can cause actin redistribution from the outer edges to the central domain of the growth cone and axon retraction through basol myosin-driven contractility (Gallo et al., 2002; Jurney et al., 2002; Meima et al., 1997a; Meima et al., 1997b).

Concomitantly with the activation of Rho, ephrin-A ligands can inhibit Rac activation in retinal and cortical neurons (Shamah et al., 2001; Wahl et al., 2000). Furthermore, the kinase Pak, a major downstream effector of Rac and Cdc42, is also inhibited by ephrin-A treatment. However, it is unclear whether Rac inactivation is a direct consequence of Rho activation or is mediated through an independent signaling pathway. Jurney et al (Jurney et al., 2002) have shown that, in cultures of retinal neurons, Rac is only briefly inactivated by ephrin-A ligands, and recovers within minutes at the onset of growth cone collapse. Indeed, Rac activity is required for reorganizing actin filaments to the center of the collapsing growth cone and for driving endocytosis of the plasma membrane. Further experiments are needed to elucidate the details of the connection between Eph receptors and the Rac signaling pathway. Remarkably, EphB receptors seem to interact with a different group of exchange factors for Rho family GTPases. EphB2 has recently been shown to associate with the exchange factors intersectin (Irie and Yamaguchi, 2002) and kalirin (Penzes et al., 2003). Intersectin activates Cdc42 and its activity is synergistically enhanced by EphB2 and WASP, an adaptor that promotes branching and elongation of actin filaments through the Arp2/3 complex (Cory et al., 2002; Hussain et al., 1999). Kalirin, an exchange factor for Rac, co-clusters with activated EphB2 and seems to localize activated Rac to sites of EphB-ephrin interaction without changing the overall level of Rac activation (Irie and Yamaguchi, 2002; Penzes et al., 2003). The intersectin-Cdc42-WASP-actin and kalirin-Rac-Pak-actin pathways have been recently proposed to regulate the EphB-receptor-mediated morphogenesis and maturation of dendritic spines in cultured hippocampal and cortical neurons (Irie and Yamaguchi, 2002; Penzes et al., 2003). It will be interesting to determine whether activation of Pak downstream of Cdc42 and Rac opposes actomyosin contractility, since Pak phosphorylates and inhibits myosin light chain kinase (Sanders et al., 1999). Perhaps activation of a Cdc42/Rac-Pak signaling pathway accounts for the lack of neurite retraction downstream of EphB receptors (Meima et al., 1997b). Furthermore, it may explain the appearance of numerous hair-like structures protruding from the cell body of neurons treated with ephrin-B1 (Meima et al., 1997b) and the ephrin-B1-dependent increase in the density of dendritic spine protrusions (Penzes et al., 2003).

Differential signaling may account for the ability of ephrin-B–EphB interactions to cause modifications to cell morphology different from those induced by ephrin-A–EphA interactions. For example, in primary cortical neurons ephrin-B1 causes growth cone collapse without causing extensive neurite retraction (Meima et al., 1997b). Furthermore, the neurites become thinner and develop large swellings along their lengths, which are classic morphological signs of microtubule depolymerization (Baas and Ahmad, 2001). Indeed, ephrin-B1 causes microtubule depolymerization (Meima et al., 1997b). By contrast, the actin filaments remain intact but are partially redistributed to the neuronal cell body. It will be interesting to determine whether these signaling differences account for the disparity in the in vivo guidance activity of EphA and EphB receptors. For example, during the establishment of visual system topography, EphA receptors mediate axon repulsion and retraction whereas EphB receptors appear to provide a stop signal limited to the growth cone (Hindges et al., 2002; Mann et al., 2002).

The Ras family
Eph receptors also regulate the activities of small GTPases of the Ras family. The best-characterized member of this family, H-Ras, activates a MAP kinase cascade culminating in the phosphorylation and activation of the Erk1/Erk2 MAP kinases (Chang and Karin, 2001; Johnson and Lapadat, 2002). Although transcriptional regulation and increased cell proliferation are major outputs of the Ras–MAP kinase pathway, this pathway is also important for cell migration, neurite outgrowth and axon guidance (Borasio et al., 1989; Forcet et al., 2002). Indeed, MAP kinases can phosphorylate cytoskeletal targets such as microtubules and microfilaments,
in addition to myosin light chain kinase (Gundersen and Cook, 1999; Klemke et al., 1997). Eph receptors negatively regulate the Ras–MAP-kinase pathway in most cell types, with a few exceptions (Elowe et al., 2001; Miao et al., 2001; Pratt and Kinch, 2002; Zisch et al., 2000). For example, EphB2 transiently downregulates H-Ras activity and MAP kinase phosphorylation and induces neurite retraction in the NG108 neuronal cell line (Elowe et al., 2001; Tong et al., 2003). Furthermore, ephrin-B2 stimulation inhibits VEGF (vascular endothelial cell growth factor) and angiopoietin-1-mediated cell migration while downregulating the Ras–MAP-kinase pathway (Kim et al., 2002). Likewise, EphA2 downregulates the Ras–MAP-kinase pathway in fibroblasts, endothelial cells, epithelial cells and tumor cells (Miao et al., 2001). Remarkably, the Eph receptors can attenuate Ras–MAP-kinase signaling downstream of other receptors, such as integrins and various families of receptor tyrosine kinase (Elowe et al., 2001; Grunwald et al., 2001; Kim et al., 2002; Miao et al., 2001). However, it remains to be determined whether this may be a result of crosstalk between activated H-Ras and Rho family proteins (Bar-Sagi and Hall, 2000) or other mechanisms.

At least in some cases, the ability of Eph receptors to regulate the Ras–MAP-kinase pathway seems to depend on the Ras GTPase-activating protein, Ras-GAP (Tong et al., 2003). Although RasGAP binds to activated EphB receptors (Becker et al., 2000; Hock et al., 1998; Holland et al., 1997; Kim et al., 2002), it is not clear whether direct physical interaction is essential (Miao et al., 2001; Tong et al., 2003). This is further complicated by the ephrin-induced association of RasGAP with p62Bosk (a negative regulator of the MAP kinase pathway) (Jones and Dumont, 1999) and the connection of RasGAP with RhoGAP (Holland et al., 1997; Leblanc et al., 1998).

R-Ras is another Ras protein whose function is suppressed downstream of Eph receptors. This relies on a novel regulatory mechanism involving tyrosine phosphorylation of the effector domain of R-Ras by EphB2 (Zou et al., 1999). R-Ras positively regulates integrin-mediated adhesion (Zhang et al., 1996), and R-Ras tyrosine phosphorylation decreases adhesion and rounding of 293 cells transfected with EphB2. This is presumably caused by interfering with the ability of R-Ras to bind effector proteins. EphB receptors can also regulate Rap1 in human aortic endothelial cells (Nagashima et al., 2002). Rap1, like R-Ras, positively modulates integrin-mediated adhesion (Caron et al., 2000; Reedquist et al., 2000). Ephrin-B1 treatment of human aortic endothelial cells, which express EphB1, causes Crk-dependent Rap1 activation and cell spreading. A possible link between Eph receptors and R-Ras/Rap1 is SHEP1. SHEP1 contains an SH2 domain that binds activated Eph receptors and a guanine nucleotide exchange factor-like domain that binds R-Ras, Rap1 (Dodelet et al., 1999) and the docking protein Cas (Sakakibara and Hattori, 2000). SHEP1 promotes Rap1 activation through a complex with Cas, the adaptor Crk, and its associated exchange factor C3G (Sakakibara et al., 2002).

Other signaling pathways downstream of Eph receptors

Eph receptors also influence other signaling molecules that regulate cell behavior. However, the information available is not yet sufficient to develop a coherent model of these Eph pathway pathways. Several Eph receptors have been reported to suppress (Miao et al., 2000; Zou et al., 1999) or promote (Huynh-Do et al., 1999; Nagashima et al., 2002; Stein et al., 1998b) integrin activity. Focal adhesion kinase (FAK), a critical element in integrin signaling, may connect Eph receptors with integrins (Miao et al., 2000). In PC-3 prostate carcinoma cells, ligand activation of EphA2 causes dissociation of FAK and the transient recruitment of the phosphotyrosine phosphatase Shp2, which dephosphorylates FAK and its substrate paxillin. This correlates with inhibition of integrin-mediated adhesion, cell spreading and cell migration. However, this mechanism may be cell-type specific because EphA2 activity can increase FAK phosphorylation in NIH3T3 cells and enhance cell spreading in a FAK-dependent fashion (Carter et al., 2002).

EphA2-mediated cell spreading on adhesive substrates depends not only on FAK, but also on Rho and the FAK-binding protein Cas (Carter et al., 2002). Cas is an adaptor that mediates assembly of structural and signaling proteins at FAK-containing integrin adhesion sites. Some of the Cas interactions depend on Cas tyrosine phosphorylation. Indeed, EphA2 activity increases Cas tyrosine phosphorylation in NIH 3T3 cells (Carter et al., 2002). Similarly, EphB1 activation in human aortic endothelial cells increases Cas tyrosine phosphorylation while also promoting an association with the Crk adaptor protein (Nagashima et al., 2002). This Cas/Crk pathway may be important for Rap1 activation in membrane ruffles after ephrin-B1 treatment. Interestingly, SHEP1 binds to both Cas and Rap1 (Dodelet et al., 1999) and may link Eph receptors and these proteins during adhesion and migration.

EphA8 has also been reported to regulate integrin function in both NIH3T3 and 293 cells through a constitutive interaction with the p110γ subunit of PI 3-kinase (Choi and Park, 1999; Gu and Park, 2001). Interestingly, it appears that EphA8 inhibits cell adhesion in 293 cells but stimulates adhesion of NIH 3T3 cells. Activation of PI 3-kinase is also important for endothelial cell migration and proliferation downstream of EphB4 (Steinle et al., 2002). The SH2 domain of the p85 regulatory subunit of PI 3-kinase also interacts with activated EphA2, but the significance of this interaction is not clear (Pandey et al., 1994).

In addition, Eph receptors can modify cell behavior by signaling through other SH2-domain-containing adaptor proteins. It was recently reported that the SH2 domain of Grb7 binds to the tyrosine phosphorylated SAM domain of EphB1 and this association can modify cell migration (Han et al., 2002; Han et al., 2001). Interestingly, this interaction appears to be selective, as Grb7 does not bind the related receptor EphB3. The same phosphorylated tyrosine motif of EphB1 and EphB2 mediates binding of the low molecular weight phosphotyrosine phosphatase (LMW-PTP), an interaction that regulates cell adhesion (Stein et al., 1998b). The adaptors She, Grb2, Grb10 and Nck can also interact with Eph receptors (Pratt and Kinch, 2002; Stein et al., 1996; Stein et al., 1998a). The Nck/NIK (Nck-interacting Ste20 kinase) pathway is important to activate JNK and upregulate integrin-mediated adhesion (Becker et al., 2000). Nck could also link Eph receptors to the regulation of the actin cytoskeleton by cooperating with Rho proteins. It interacts with downstream effectors of Rac and Cdc42, including Pak3 and WASH, which influence actin polymerization (Becker et al., 2000; Holland et al., 1997). Additionally, some Eph receptors seem to
selectively bind the ubiquitin ligase and adaptor protein Cblc. EphB6 clustering in Jurkat cells leads to Cbl dephosphorylation (Freywald et al., 2002; Luo et al., 2001). Cbl may couple EphB6 to Grb2 and Crk, and promote EphB6 degradation via the proteasome pathway. The interaction of Cbl with EphA2 instead occurs only after ephrin binding and promotes activation-dependent EphA2 degradation (Walker-Daniels et al., 2002; Wang et al., 2002a).

A number of additional signaling proteins have been linked to Eph receptor downstream signaling pathways. For example, the Src and Abl family cytoplasmic tyrosine kinases associate with activated Eph receptors and this may contribute to the regulation of cytoskeletal organization, cell migration and axon guidance (Kalo and Pasquale, 1999). Dominant negative approaches have suggested that signaling by the Src family kinase Fyn plays an important role in linking EphA8 signaling to cell attachment responses (Choi and Park, 1999). Interestingly, while Eph receptors are thought to activate Src proteins, the EphB2 receptor inhibits the in vitro activity of Abl (Yu et al., 2001; Zisch et al., 1998).

Reverse signaling

Forward signaling is clearly an important mechanism used by Eph receptors to modify cell behavior. However, the Eph receptors also have kinase-independent, cell non-autonomous functions and can activate ‘reverse’ signaling through their ephrin ligands (Henkemeyer et al., 1996; Kullander et al., 2001). Thus, the Eph receptors and ephrin ligands communicate bi-directional signals upon their engagement (Fig. 2).

The first studies to suggest that the ephrins are more than merely ligands for the Eph receptors were in the mid 1990s, when Brambilla et al. found that the cytoplasmic portion of ephrin-B1 can negatively regulate transformation of NIH-3T3 cells transfected with EphB-Trk chimeric receptors (Brambilla et al., 1995). This was followed by two seminal reports showing that the extracellular portions of EphB receptors can induce ephrin-B tyrosine phosphorylation (Bruckner et al., 1995; Holland et al., 1996). Src family kinases are responsible for ephrin-B phosphorylation upon Eph receptor engagement (Holland et al., 1996; Palmer et al., 2002), and three conserved tyrosines in the cytoplasmic domain of ephrin-B1 have been identified as in vivo phosphorylation sites (Kalo et al., 2001). This suggests a receptor-like property for ephrin-B molecules, and the possibility of transmitting signals through proteins containing SH2 domains.

At least one SH2-domain-containing protein binds to tyrosine-phosphorylated ephrin-B1 (Cowan and Henkemeyer, 2001). The adaptor protein Grb4, which has three SH3 domains and an SH2 domain, could link ephrin-Bs to a vast signaling network that modifies cell morphology through reorganization of the actin cytoskeleton. Indeed, activation of ephrin-B1 increases Fak activity, redistributes the Fak-binding protein paxillin, and leads to disassembly of focal adhesions. A mechanism that may serve to turn off phosphorylation-dependent ephrin-B reverse signals involves the delayed recruitment of the phosphotyrosine phosphatase PTP-BL, which can dephosphorylate the ephrin-B cytoplasmic domain and inactivate Src family kinases (Palmer et al., 2002). The interaction of PTB-BL with ephrin-B1 is mediated by the PDZ domain of the phosphatase, which binds the C-terminal PDZ-binding motif of ephrin-B molecules. Interestingly, phosphorylation of this region may modulate ephrin-B binding to PDZ domain-containing proteins.

Ephrin-Bs can also initiate reverse signaling through PDZ-domain-mediated associations (Lu et al., 2001). The GTPase-activating protein PDZ-RGS3, which catalyzes the hydrolysis of GTP to GDP in the α subunits of heterotrimeric G proteins, binds to the PDZ-binding motif of ephrin-B molecules. Signaling through PDZ-RGS3 mediates the de-adhesion of Xenopus embryo cells expressing ephrin-B1. It also inhibits SDF1 (stromal cell derived factor 1)-mediated cerebellar granule cell chemotaxis through the CXCR4 G-protein-coupled chemokine receptor. During cerebellar development, ephrin-B activation by EphB receptors may attenuate granule cell attraction to SDF-1, which is expressed at the pial surface, and allow cells to migrate from the external to the internal granule cell layer (Lu et al., 2001). This signaling mechanism may have broad implications for cell migratory behavior in other systems as well.

In vivo evidence also supports the importance of reverse signaling through ephrins. Intriguingly, replacement of the wild-type receptor with a signaling-deficient mutant can rescue the axon guidance defects observed in several knockout lines (Birgbauer et al., 2000; Henderson et al., 2001; Henkemeyer et al., 1996; Kullander et al., 2001). Furthermore, removal of the ephrin-B2 cytoplasmic domain in mice causes vascular defects similar to those found in ephrin-B2-knockout mice (Adams et al., 2001). This suggests that reverse signaling is essential for sculpting the developing vasculature. Ephrin-B signaling also promotes neovascularization in a corneal micropocket assay, and enhances endothelial cell attachment and migration (Huynh-Do et al., 2002). These effects are believed to be mediated through integrin signaling and are accompanied by JNK activation. The PDZ-domain-binding site of ephrin-B1, in particular, appears to be important for this function.

Ephrin-B signaling is also necessary for boundary formation. In Zebrafish animal cap studies, the bi-directional signaling through ephrin-B2 and EphB2 restricts cell intermingling, while unidirectional signaling through either ephrin-B2 or EphB2 prevents cell communication through gap junctions. The formation of rhombomeres also relies on the activation of ephrin signaling (Mellitzer et al., 1999; Xu et al., 1999). This could be accomplished through Ephrin-induced modifications of adhesive or migratory properties of cells during development. Thus, concomitant activation of an Eph receptor and its ephrin ligand is likely to be necessary for cellular organization and maintenance in many tissues.

Ephrin-A ligands can also convey reverse signals that modify cell behavior. The ephrin-A molecules, like many GPI-anchored proteins, are targeted to lipid rafts, where they presumably assemble into protein complexes that transduce intracellular signals. Indeed, clustering of ephrin-A molecules with EphA-Fc fusion proteins recruits the Src family kinase Fyn to lipid rafts (Davy et al., 1999). This is accompanied by the redistribution of vinculin, activation of MAP kinase, tyrosine phosphorylation of a 120 Kd lipid raft protein, and increased cell substrate adhesion (Davy et al., 1999; Huai and Drescher, 2001). Interestingly, in C. elegans the primary
function of the only Eph receptor, Vab-1, may be to activate reverse signals through GPI-linked ephrins (Wang et al., 1999).

Ephrin-A reverse signals may be modulated by cell surface shedding of the ligand through its association with the metalloprotease Adam10/Kuzbanian (Hattori et al., 2000). Upon binding of EphA receptors, Adam10 cleaves ephrin-A2 from the cell surface. This could serve a dual function. Ephrin-A cleavage from the cell surface allows Eph-receptor-bearing structures such as growth cones to change their response to ephrin-A molecules from adhesion to repulsion. In addition, the cleaved ligand is no longer able to transmit signals or activate EphA receptors, and hence both reverse and forward signaling is terminated.

Crosstalk
Adding to the complexity of Eph-receptor–ephrin bi-directional signaling is the ability of Eph proteins to communicate with a variety of other cell surface proteins. This crosstalk may allow the Eph receptors and ephrins to broaden their repertoire of functions. An example of a protein that may collaborate with EphB receptors during embryonic development is Ryk, a catalytically inactive orphan receptor tyrosine kinase (Katso et al., 1999). Ryk associates with EphB2 and EphB3 and all three receptors bind the cell-junction-associated PDZ-domain-containing protein AF6 through their C-termini. EphB2 and EphB3 may signal through Ryk by causing Ryk tyrosine phosphorylation. However, murine but not human Ryk is susceptible to this phosphorylation, indicating species-specific differences in the crosstalk between Eph receptors and Ryk (Trivier and Ganesan, 2002). Intriguingly, Ryk-deficient mice have a cleft palate, which is similar to EphB2/EphB3 double-knockout mice and suggests that these proteins cooperate during palate formation (Halford et al., 2000).

Another potentially interesting form of crosstalk is that of EphB receptors with the multi-transmembrane protein ARMS (ankyrin repeat-rich membrane spanning). ARMS associates with the p75 and Trk nerve growth factor receptors, and is a substrate of both Eph and Trk receptors (Kong et al., 2001). It will be interesting to determine whether ARMS links the Eph and nerve growth factor receptor signaling pathways during axon outgrowth, synaptogenesis and plasticity.

More recently, EphB receptors have been shown to associate directly with NMDA receptors at synapses. Ephrin-B-induced activation of EphB receptors causes NMDA receptor clustering, potentially helping to initiate the development of the postsynaptic specialization (Dalva et al., 2000). This interaction is functionally important because EphB2 activation enhances Src-mediated NMDA receptor phosphorylation, which results in increased glutamate-induced calcium influx through the NMDA receptor (Takasu et al., 2002). This is consistent with the reduced NMDA-mediated currents in EphB2 knock out mice (Henderson et al., 2001). Thus, crosstalk between Eph and NMDA receptors could be important for early events during synaptogenesis and in modifying the physiological properties of synapses.

Clues are also beginning to emerge that some Eph receptors form complexes with other Eph receptors. An example is EphB6, which has no detectable catalytic activity but forms a hetero-receptor complex with EphB1 and is transphosphorylated as a result (Freywald et al., 2002; Gurniak and Berg, 1996; Matsuoka et al., 1997). EphB6 also seems to convey signals through association with other types of cell surface receptor. Antibody-mediated clustering of EphB6 in Jurkat cells results in the co-clustering of the T-cell receptor and promotes the proliferation of normal T cells (Luo et al., 2002). Furthermore, EphB6 enhances the production of certain lymphokines and promotes Fas-mediated apoptosis when co-clustered with the T-cell receptor (Luo et al., 2001). These studies suggest that EphB6 serves to reduce the threshold for T-cell receptor activation in lymphocytes. Thus, even in the absence of kinase activity, Eph receptors can communicate with each other and with other cell surface proteins to modify signaling pathways.

Ephrins may also exhibit crosstalk with receptor tyrosine kinases. For example, the PDGF receptor phosphorylates the ephrin-B cytoplasmic domain (Bruckner et al., 1997), and the activated FGF receptor associates with Xenopus ephrin-B1 and phosphorylates it (Chong et al., 2000). Interestingly, phosphorylation by the FGF receptor reverses the effects of ephrin-B1 on cell dissociation in Xenopus embryos (Chong et al., 2000). Ephrin-B1 is also a substrate of the Tie-2 receptor, at least in vitro (Adams et al., 1999).

Perspectives
Recent advances have clarified many aspects of Eph receptor and ephrin bi-directional signaling, but have also raised several perplexing issues. A pressing question to be answered is how Eph proteins can have distinct and sometimes opposite effects on cell behavior. Many avenues will have to be investigated to explain these differences (Holmberg and Frisen, 2002). Another intriguing issue is the extent of divergence in the Eph signaling pathways, and whether different Eph proteins can be interchangeable. The years to come will reveal the full extent of the influence of Eph proteins on biological processes, and perhaps this knowledge will translate into useful tools for disease intervention.

The work in the authors’ laboratory is supported by grants from NIH and the Department of Defense.

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