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The Eph family: a multitude of receptors that mediate cell recognition signals

Andreas H. Zisch, Elena B. Pasquale

The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla CA 92037, USA

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Abstract. The Eph receptor tyrosine kinases are emerging as molecules that guide the migration of cells and growth cones during embryonic development. Based on their concentration in embryonic regions containing growing neuronal processes, the Eph receptors were suspected early on to have a role in regulating aspects of axon growth. The most distinctive role of the Eph receptors appears to be their ability to mediate cell-cell repulsion through the binding of a ligand on an adjacent cell surface. The repulsive interactions are presumably mediated by transient receptor activation at the boundaries of complementary regions of high ligand or receptor expression. In contrast, overlapping expression patterns may regulate cell adhesion and cytoskeletal organization with possible consequences on the overall growth and fasciculation of neuronal processes. A notable feature of Eph receptor signaling is that, upon receptor binding, responses may also be elicited in the ligand-expressing cells. A better understanding of Eph receptor function requires the elucidation of their signaling properties. Recent evidence suggests a functional interaction between the Eph receptor EphB2 and neural cell adhesion molecules of the L1 family, which have well-recognized roles in the formation of neuronal projections. Only a few cytoplasmic signaling molecules that bind to the activated Eph receptors have been identified. Several of these molecules are known to transduce signals regulating cytoskeletal organization and neurite outgrowth. It is currently unclear why there is a need for fourteen distinct Eph receptor genes, many of which appear to encode several variant forms with distinct functional properties, but it is tempting to speculate that such diversity is necessary to refine the spatial organization of embryonic structures.

Key words: Receptor tyrosine kinase – Axon pathfinding – Neural development – L1 – Src family kinases

Introduction

A number of receptor tyrosine kinases mediating mitogenic signals in non-neuronal cells are also highly expressed in neural tissues, where they have been shown to play important roles in the differentiation and maintenance of cells with a neuronal phenotype. These tyrosine kinases include members of the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin receptor families, and others that are less well characterized. In contrast, the most distinctive roles identified for the Eph family in the nervous system are the steering of growth cones and the regulation of axon fasciculation in concert with their ligands (Drescher et al. 1995; Cheng et al. 1995; Winslow et al. 1995). The ligands for the Eph receptors, or ephrins, are membrane-anchored molecules, bound to the cell surface through a glycosyl-phosphatidyl-inositol (GPI)-linked moiety (ephrin-A subclass) or a transmembrane segment (ephrin-B subclass). It is conceivable that the Eph receptors influence the directional migration of growth cones not because they activate signaling pathways that are unique to molecules involved in guidance, but because their activating ligands, which are anchored to cell surfaces, are precisely localized to specific regions. Although the available information is still incomplete, most cytoplasmic signaling molecules that bind to activated Eph receptors are well-known components of signaling pathways of other receptor tyrosine kinase families and adhesion molecules. The Eph receptors interact with their ligands in a mode that had been previously attributed to molecules that mediate cell-cell adhesion: the interaction between an Eph receptor in the neuronal growth cone or axon and its cognate ligand on the surface of an apposing cell is an interaction between two membrane-bound recognition molecules. The cell-cell contact

* In this review, we have adopted the new unified nomenclature for Eph receptors and their ligands (Eph Nomenclature Committee 1997)

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Correspondence to: E.B. Pasquale (Tel.: +1-619-455-3131; Fax: +1-619-646-3199; e-mail: elenap@ljcrf.edu)

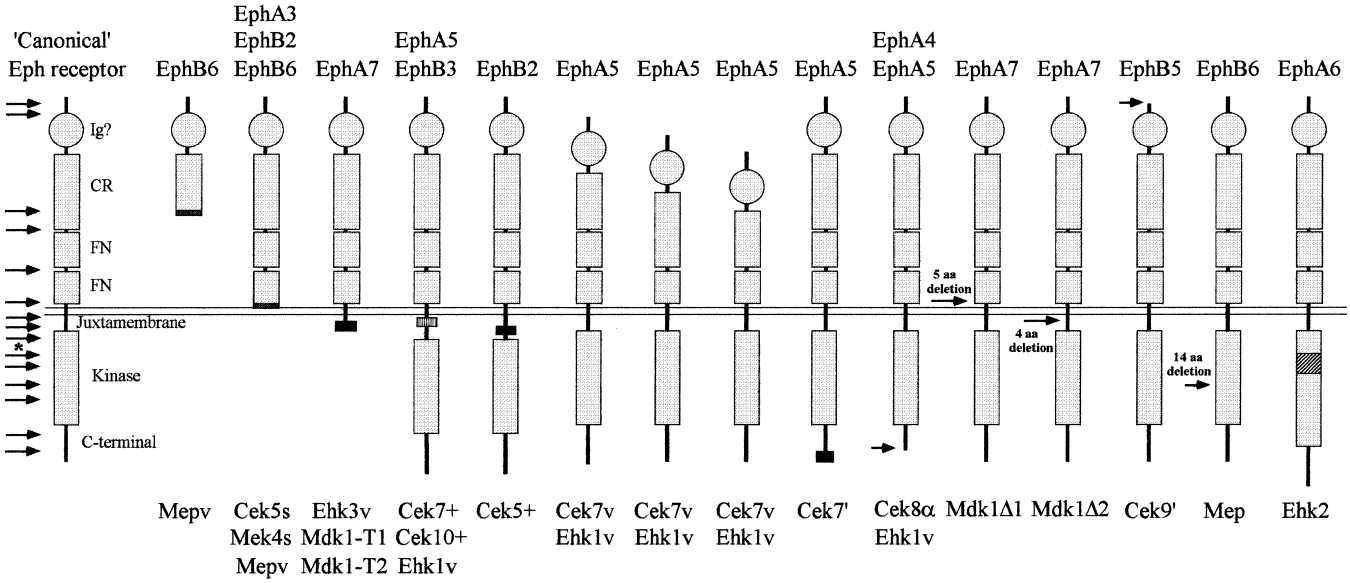

Fig. 1. Variant forms of Eph receptors. The structure of a "canonical" Eph receptor is shown *left*, and the positions of the introns identified in the EphB2 gene (Connor and Pasquale 1995) are marked with *arrows*. An *asterisk* marks the position of an intron that is present in EphA1 (Maru etal. 1988), but not EphB2. *Ig*, Immunoglobulin-like domain; *CR*, cysteine-rich region; *FN*, fibronectin type III repeat. Receptor names according to the new nomenclature (Eph Nomenclature Committee 1997) are indicated *top*; original names of variant forms are indicated *bottom*. Variant forms that have not been previously named are indicated with a *v* after the original name of the receptor. References for the variant forms are: Mep variants (Gurniak and Berg 1996), Cek5s (Connor and Pasquale 1995), Mek4s (Sajjadi etal. 1991), Ehk3v (Valenzuela etal. 1995), Mdk1 variants (Ciossek etal. 1995), EphA5 variants (Siever and Verderame 1994), Ehk1 variants (Maisonpierre etal. 1993), Cek8α (Ohta etal. 1996), Cek9' (Soans etal. 1996). Only one form was isolated for Ehk2 (Maisonpierre etal. 1993); it contains an insertion at a position corresponding to the location of the intron in EphA1 that is not conserved in EphB2. Mep contains a 14 amino-acid (*aa*) deletion at a position corresponding to the location of an intron in EphB2

is then believed to trigger the initiation of signaling cascades leading to local effects in growth cones and axons. It is not yet known whether signals mediated by Eph receptors can also reach the nucleus and influence gene expression.

Structural diversity of the Eph receptor gene family

The Eph receptors represent a very large family of receptor tyrosine kinases with high expression in embryonic tissues. For this reason, many Eph receptors have been identified from embryonic cDNA libraries. We have cloned chicken EphA3 (Sajjadi et al. 1991), EphB2 (Pasquale 1991), EphA4, EphA5, EphB1, EphB3, and EphB5 (Sajjadi and Pasquale 1993) in a systematic search for tyrosine kinases epressed in chicken embryonic tissues. Fourteen different Eph receptor genes have been identified to date in different species, including mammals, birds, frogs, fish, and invertebrates (Friedman and O'Leary 1996), and additional Eph receptor genes may yet exist. The conservation of these genes throughout evolution attests to their importance in fundamental biological processes.

Comparing the first Eph receptor sequences identified revealed their characteristic structural domains (O'Bryan et al. 1991; Pasquale 1991). The Eph receptors are signaling molecules with an extracellular domain that contains motifs typically found in adhesion molecules. This region contains a putative immunoglobulin domain at the amino-terminus, a central cysteine-rich region, and two fibronectin type III repeats near its single membrane spanning segment. Within the cysteine-rich region, there are two C*n*C*x*C*n*C repeats (where C represents cysteine, *n* a stretch of 12–15 amino acids, and *x* any amino acid), which are most similar to sequences found in the EGF repeats of extracellular matrix proteins, such as fibrillin, tenascin, and thrombospondin (Connor and Pasquale 1995). The cytoplasmic region of the Eph receptors contains a conserved tyrosine kinase domain flanked by a less conserved juxtamembrane region and carboxy-terminal tail (Sajjadi and Pasquale 1993).

As more sequences have been identified, a number of variant forms of the Eph receptors has also been discovered (Fig. 1). Several of these variant forms, which presumably have different functional properties, have been demonstrated to originate by alternative processing. For example, truncated forms comprising the extracellular domains of the receptors EphA3 and EphB2 are generated by alternative polyadenylation (Sajjadi et al. 1991; Connor and Pasquale 1995). These truncated forms are expected to bind ligands but not to transduce signals and may downregulate activation of the full-length receptors or upregulate signaling through the cytoplasmic domain of ligands (Holland et al. 1996; Brückner et al. 1997; see below). Another variant form of EphB2, EphB2+, contains an insertion in the juxtamembrane domain, which originates by the alternative use of distinct 5'

splice sites. The insertion in EphB2+ contains potential phosphorylation sites that may modulate the activity of the receptor or serve as additional binding sites for signaling molecules (Connor and Pasquale 1995). Although EphB2+ and the truncated forms of EphB2 and EphA3 appear to be generally expressed at lower levels than the non-variant forms of these receptors (Sajjadi et al. 1991; Connor and Pasquale 1995; Holash and Pasquale 1995), they may nevertheless play essential roles.

Variant forms of Eph receptors with insertions, deletions, or substitutions, which are predicted to arise by alternative processing from genes that have exon/intron organizations similar to the EphB2 gene, have been reported (Fig. 1), and multiple mRNA species have been detected for many of the Eph receptors, particularly in the nervous system (Maisonpierre et al. 1993; Sajjadi and Pasquale 1993; Siever and Verderame 1994; Taylor et al. 1994; Ciossek et al. 1995; Valenzuela et al. 1995). As we begin to understand the functions of the Eph receptors, we should keep in mind that alternative splicing and polyadenylation could have important functional implications that need to be further elucidated.

The expression patterns of the Eph receptors in the nervous system suggest that they regulate aspects of axon growth

The expression patterns of the Eph receptors provided the first clues to their functions. An initial assessment of the overall distribution of chicken Eph receptors in various tissues by Northern blotting revealed high expression in the embryo, particularly in the brain, and persistent expression in adult neural tissues (Sajjadi and Pasquale 1993; Van der Geer et al. 1994). Localization of the Eph receptors at the protein level provided the first indication of their role in axon outgrowth. The first Eph receptor to be precisely localized in neural structures was EphB2 (Pasquale et al. 1992), which was found to be highly expressed in regions rich in nerve cell processes and fasciculating axons.

In the developing chicken cerebellum, EphB2 immunoreactivity is concentrated in the molecular layer, associated with the axons of postmitotic granule cells (parallel fibers). Although the molecular layer of the cerebellum is very rich in synaptic connections, EphB2 is concentrated in the fasciculated portions of the axons of granule neurons, but not in local enlargements corresponding to synapses onto Purkinje cells. This localization, together with the adhesion motifs present in the EphB2 extracellular region, suggests that EphB2 interacts with other cell-surface associated molecules that are known to be involved in the formation of the cerebellar cortical layers (see below) and that EphB2 may regulate the growth, guidance, and/or bundling of certain unmyelinated axonal processes. Another Eph receptor, EphA4, is also concentrated in the molecular layer of the cerebellum. EphA4 is found in the dendrites of cerebellar Purkinje cells, but interestingly not in postsynaptic densities (Martone et al. 1997). Based on these findings, it is tempting to speculate that EphB2 and EphA4 guide neuronal processes during the initial contacts between the axons of granule neurons and the dendrites of Purkinje cells; regions devoid of Eph receptors may mark sites destined to become synapses.

In the chicken retina, at least two Eph receptors, EphB2 and EphB5, are highly concentrated in the short range neuronal projections of the plexiform layers (Pasquale et al. 1994; Soans et al. 1996). EphA4, EphB2, and EphB5 have also been found to be concentrated in vivo in the axons of retinal ganglion cells, which form the optic nerve and project to the optic tectum (Holash and Pasquale 1995; Soans et al. 1996). EphA3, EphA4, EphA5, and EphB2 have also been detected in the axons and growth cones of retinal ganglion cells in culture (Holash and Pasquale 1995; Monschau et al. 1997). In the spinal cord regions, the Eph receptors EphA2, EphA4, and EphB2 have all been detected in spinal nerves (Henkemeyer et al. 1994; Soans et al. 1994; Magal et al. 1996; Ohta et al. 1996, unpublished). Taken together, these expression patterns suggest that the Eph family regulates aspects of axon growth in various regions of the developing nervous system.

Reciprocal expression patterns of Eph receptors and their ligands are consistent with repulsive signaling pathways

Once the ligands for the Eph receptors were identified, their expression patterns could be compared with those of the receptors by in situ hybridization or by using receptor ectodomains as probes (Cheng and Flanagan 1994; Flenniken et al. 1996; Gale et al. 1996a). Conversely, ligand ectodomains were used to localize receptor expression. In many instances, the expression patterns of ligands and receptors appeared complementary. Transient receptor activation at the boundaries of distinct domains of ligand and receptor expression are consistent with repulsive interactions and may reflect a role in restricting cell or axon migration. An example of this are the complementary distributions of the EphB2 receptor in the ventral 8-day chicken retina and the ephrin-B1 ligand in the dorsal retina (Holash et al. 1997). These distributions are consistent with a repulsive role of ligand-expressing cells toward receptor-expressing cells and may be important in the compartmentalization of the retina into a ventral and a dorsal region. EphB2 is also expressed in the axons of retinal ganglion cells from the ventral retina, these axons are segregated in about half of the optic nerve (Holash and Pasquale 1995). If ephrin-B1 is localized in the axons of retinal ganglion cells from the dorsal retina, repulsive or even adhesive (see below) interactions between EphB2 and ephrin-B1 may regulate the topography, fasciculation, or growth rate of axons growing in the optic nerve toward the optic tectum.

Compartments of ligand expression adjacent to compartments of receptor expression have been reported in other regions of the embryo. In the differentiating limbs, for example, ephrin-A ligands are concentrated in the interdigital zone, whereas their receptors are concentrated within the forming cartilagenous digits (Flenniken et al. 1996; Gale et al. 1996a). In the hindbrain, the receptors

EphA4, EphB2, and EphB3 are concentrated in rhombomeres 3 and 5 (Gilardi-Hebenstreit et al. 1992; Becker et al. 1994; Henkemeyer et al. 1994), whereas the transmembrane ligands ephrin-B1, ephrin-B2, and ephrin-B3 are concentrated in the adjacent rhombomeres 2, 4, and 6 (Bergemann et al. 1995; Flenniken et al. 1996; Gale et al. 1996b). Misexpression in *Xenopus* and zebrafish hindbrain of a dominant negative form of EphA4 lacking the kinase domain caused blurring of the rhombomere boundaries (Xu et al. 1995). These results indicate that the interaction between Eph receptors and their ligands are important for maintaining well-defined boundaries between the separate anatomical compartments of rhombomeres.

In some cases, regions at which a ligand is concentrated appear to be avoided by axons that express high levels of the cognate Eph receptor, resulting in reciprocal expression patterns. In the developing optic tectum, the posterior portion, which contains high levels of the ligands ephrin-A2 and ephrin-A5, is avoided by axons expressing the receptor EphA3 (Cheng et al. 1995; Drescher et al. 1995; see also other contributions to this issue). In vitro and in vivo experiments have confirmed the repulsive activity of ephrin-A2 and ephrin-A5 ligands toward axons of temporal retinal ganglion cells, which express the EphA3 receptor (Drescher et al. 1995; Nakamoto et al. 1996; Monschau et al. 1997). In the hippocamposeptal system, the ventral lateral septum, which expresses the ligands ephrin-A2, ephrin-A3, and ephrin-A5, is avoided by axons from the hippocampus that express the receptor EphA5 (Zhang et al. 1996). In the developing somites, the caudal halves, which express the transmembrane ligands ephrin-B1 or ephrin-B2, are avoided by the axons of spinal motor neurons, which express Eph receptors that interact with these ligands and travel through the rostral halves of the somites (Wang and Anderson 1977). In vitro experiments have confirmed the repulsive effects of the transmembrane ligands toward receptor-bearing axons extending from neural tube explants (Wang and Anderson 1997).

It has recently been reported that ephrin-B ligands have receptor-like signaling potential (Holland et al. 1996; Brückner et al. 1997). Upon contact with Eph receptor ectodomains, they become phosphorylated on tyrosine, presumably through an associated tyrosine kinase. Contact between receptor-expressing cells and ligand-expressing cells results in the phosphorylation on tyrosine of both receptor and ligand, suggesting that bidirectional signaling occurs. The mirror-image distributions of EphB2 and ephrin-B1 in the 8-day chicken embryonic retina and the complementary distributions of Eph receptors and ephrin-B ligands in the hindbrain are consistent with the hypothesis that signaling pathways become activated not only in receptor-expressing cells, but also in ligand-expressing cells. Indeed, tyrosine phosphorylation of transmembrane ligands has been detected in vivo in 10-day and 12-day mouse embryos. It is not known whether the signals transmitted by the ligands are also repulsive.

Genetic evidence also suggests that the transmembrane ligands can transmit signals (Henkemeyer et al. 1996). In mice that are deficient in the EphB2 receptor, the axons of the posterior portion of the anterior commissure project abnormally, whereas in mice that express a form of EphB2 containing the extracellular domain and lacking the kinase domain, the anterior commissure appears normal. A possible explanation of this surprising finding is that the EphB2 extracellular portion acts as a guidance cue and activates signaling pathways in ligand-expressing axons of the anterior commissure. Indeed, the EphB2 ligand ephrin-B1 is expressed in the axons of the posterior portion of the anterior commissure; these axons are surrounded by regions of high receptor expression.

Instances of elevated Eph receptor phosphorylation indicate regions of extensive interaction with a ligand

Recent evidence suggests that the repulsive activities mediated by the Eph receptors require a discontinuous presentation of the ligand (Nakamoto et al. 1996; Wang and Anderson 1997). However, Eph receptors and their ligands are not always segregated to different anatomical regions. Several Eph receptors exhibit substantial phosphorylation on tyrosine in embryonic tissues; this probably reflects the co-localization and extensive interaction of receptor and ligand. For example, EphB5 is phosphorylated on tyrosine in many tissues of the developing chicken embryo, particularly during the first half of embryonic development (Soans et al. 1996), and EphB2 phosphorylation on tyrosine increases during retinal differentiation (Pasquale et al. 1994). Although the ligand for EphB5 has not yet been identified, upregulation of EphB2 phosphorylation in the retina correlates with an overlap of EphB2 and ephrin-B1 expression at both the mRNA and protein levels. Interestingly, both EphB2 and ephrin-B1 proteins are concentrated in the forming inner plexiform layer of the retina (Holash et al. 1997). The level of resolution obtained so far in in situ hybridization and light microscopy experiments, however, has not been sufficient either to determine whether EphB2 and ephrin-B1 mRNAs are present in the same cells, or to localize precisely the proteins within neuronal processes. It is possible that subcellular microdomains of separate ligand and receptor expression exist and that many transient repulsive interactions affect the targeting of short range projections within the inner plexiform layer and contribute to the formation of neuronal circuits. Such interactions would account for the observed elevated EphB2 phosphorylation.

Alternatively, high overall receptor phosphorylation (activation) may reflect a role for Eph receptors that is unrelated to the steering of axons. The Eph receptors could be involved, directly or indirectly, in neurite outgrowth and/or the modulation of cell-cell interactions at times of neuronal migration and differentiation, when the interactions between cells are required to be plastic. As a consequence, the phosphorylation of substrates by persistently activated Eph receptors may be instrumental in achieving developmental plasticity in certain areas of the developing nervous system. A soluble form of the ligand, ephrin-A1, has been shown to enhance neuronal survival and neurite outgrowth in cultured rat spinal cord neurons, including motor neurons (Magal et al. 1996). It is presently unclear whether the biological effects of Eph

ligands in soluble form differ from those of membrane-anchored ligands. Whether ligands for the Eph receptors are present as soluble molecules in vivo remains to be determined, although in principle the GPI-linked ligands may be released in the extracellular space as soluble proteins by phosphatidylinositol-specific phospholipases, and the extracellular portions of the transmembrane ligands may be cleaved by proteases. Soluble ligands have indeed been detected in cell culture supernatants (Bartley et al. 1994; Winslow et al. 1995; Böhme et al. 1996).

It is also possible that adhesive properties of Eph receptors and their ligands play a physiological role in regions where they are extensively co-localized. Formation of stable cell–cell contacts has been observed between cells expressing the ligand ephrin-B1 and cells expressing the receptor EphB3 (Böhme et al. 1996). Furthermore, the ectodomain of EphB2, when coated on a surface, mediates the attachment of ephrin-B1-expressing cells and conversely, ephrin-B1 coated on a surface binds EphB2-expressing cells (Holash et al. 1997). The adhesive ability of Eph receptors and their ligands has been instrumental in isolating the ligands ephrin-B1 and ephrin-A2 by expression-cloning approaches (Shao et al. 1994, 1995).

The signaling pathways of the Eph receptors include molecules known to regulate neurite outgrowth

Activation of Eph receptors could lead, directly or indirectly, to the phosphorylation on tyrosine of structural proteins within axons. Phosphorylation of these proteins may modulate the assembly of microtubules or microfilaments, thereby affecting the formation and growth of axonal processes or the transport of molecules or vesicles along axons. The function of neuronal cell adhesion molecules may also be influenced by the activation of Eph receptors. The spatially restricted activation of Eph receptors would result in axon guidance, whereas a more widespread activation may influence overall neurite outgrowth and/or fasciculation and neuronal survival. To understand the way in which Eph receptors function at the molecular level, we need to elucidate the signaling pathways of each receptor and its variant forms.

"Crosstalk" between the EphB2 receptor and neural cell adhesion molecules of the L1 family

For at least some proteins that have been previously regarded as "classical" adhesive molecules, it has become evident that they do not simply mediate adhesion between surfaces of neighboring cells or to the extracellular matrix, but that they also act as signaling receptors. The signaling activities of cell adhesion molecule are acquired through their specific functional association with kinases, which may also phosphorylate the adhesion molecule itself. In association with kinases, adhesion molecules may form recognition complexes on the cell surface that trigger signals in response to changes in the environment.

Searching for target molecules of the EphB2 receptor, we have considered the transmembrane L1 family glycoproteins (see Wong et al. 1995 for a review; Hlavin and Lemmon 1991) as interesting candidate proteins (Zisch et al. 1997). Based on their expression in axons and growth cones in the developing central and peripheral nervous system, L1 family proteins have been implicated in cell-cell recognition events during neuronal pattern formation. A number of studies have demonstrated roles for L1 family proteins in neuronal migration, neurite outgrowth, axon fasciculation, synapse formation, and myelination during embryonic development (Lindner et al. 1983; Stallcup and Beasley 1985; Fischer et al. 1986; Kuhn et al. 1991; Williams et al. 1992; Rose 1995). The L1 protein is crucial for normal brain development, since mutations in the human L1 gene result in severe malformations of the developing brain (Rosenthal et al. 1992; Vits et al. 1994; Jouet et al. 1994). One of the phenotypic traits associated with the malfunction of L1 is agenesis of the corpus callosum. A similar defect has recently been reported in mice that are deficient in the Eph receptors EphB2 and EphB3 (Henkemeyer et al. 1996; Orioli et al. 1996). Future investigations should determine whether this is coincidence or whether it reflects a functional link between Eph receptors and L1 in vivo.

The phosphorylation of L1 family proteins has been previously reported, but only on serine residues (Salton et al. 1983; Sadoul et al. 1989; Wong et al. 1996a, b). Recently, we have demonstrated that tyrosines in the cytoplasmic portion of L1 family members are also sites of phosphorylation both in vivo and in vitro (Zisch et al. 1997). In embryonic day-13 chicken retina, both the L1 family member neuron-glia cell adhesion molecule (Ng-CAM) and EphB2 are phosphorylated on tyrosine. In support of a broad occurrence of in vivo tyrosine phosphorylation of L1 family proteins is the finding that L1 is also phosphorylated on tyrosine in 8-day old mouse brain (Heiland et al. 1996). Furthermore, phosphoamino-acid analysis of metabolically labeled L1 preparations from cultured mice cerebellar neurons suggests that the extent of L1 phosphorylation on tyrosine is comparable to the extent of serine phosphorylation. Notably, the cerebellum is also a site of high EphB2 expression (Pasquale et al. 1992), and cultured rat cerebellar neurons express activated EphB2 (A.H. Zisch, W.B. Stallcup, and E.B. Pasquale, unpublished).

To examine whether L1 phosphorylation on tyrosine correlates with the presence of activated EphB2, we transiently transfected EphB2 into B28 rat glioblastoma cells stably transfected with human L1. Expression of constitutively activated EphB2 in these cells resulted in an increase in tyrosine phosphorylation of only a limited number of proteins. Immunoblotting with anti-phosphotyrosine antibodies demonstrated that, in the presence of the activated EphB2, the L1 protein was indeed phosphorylated on tyrosine. Phosphorylation of L1 may have resulted from direct phosphorylation by EphB2, especially since tyrosines in a bacterially expressed cytoplasmic domain of L1 are excellent in vitro substrates of immunopure EphB2 (Zisch et al. 1997).

FGF receptor 1 has also been identified as a crucial component of the L1 signaling pathway, following the observation that cell-cell contact-dependent neurite out-

growth mediated by several cell adhesion molecules, including L1, requires the activation of second messenger pathways that are in turn dependent on the function of an upstream tyrosine kinase (Williams et al. 1994a; Doherty and Walsh 1996; Hall et al. 1996). Binding between L1 molecules on apposing cell surfaces is proposed to result in a physical cis-interaction between the L1 portein and FGF receptor 1, thus causing the activation of the FGF receptor signaling cascade (Williams et al. 1994a, c). This cascade involves the generation of diacylglycerol by phospholipase Cγ (PLCγ), its conversion to arachidonic acid by diacylglycerol lipase, and a subsequent influx of calcium. Interestingly, we have identified PLCγ as a candidate downstream component of the signaling pathways of the Eph receptors EphB2 and EphA4 (unpublished). The diverse range of functions proposed for the L1 protein during the establishment of the nervous system makes a strict dependence of L1 function on FGF receptor 1 seem unlikely. Perhaps in some instances, the interaction of L1 with EphB2 represents an alternative to the interaction with FGF receptor 1. In this regard, it will be important to determine whether L1 and EphB2 are colocalized in regions of the nervous system lacking FGF receptor 1. Tyrosine phosphorylation of L1 upon its interaction with FGF receptor 1 or other receptor tyrosine kinases has not been investigated, and it remains to be determined whether the ability of EphB2 to phosphorylate L1 is unique.

The implication of Eph receptors in the phosphorylation of L1 adds to the growing evidence that L1 family proteins function at least in part by associating with kinases. Phosphorylation of the cytoplasmic domain of L1 family proteins may be important in regulating axon fasciculation, since axon fasciculation in cultured chicken retinal explants is highly sensitive to drug-induced changes in cytoplasmic protein phosphorylation (Cervello et al. 1991). The functional consequences of L1 phosphorylation are presently unknown, but clues may be obtained from studies on the interaction of L1 proteins with serine kinases (Wong et al. 1996a, b), FGF receptor 1 (Williams et al. 1994b), and Src-family kinases (Kunz et al. 1996). The cytoplasmic serine/threonine kinase $p90^{rsk}$ has been shown to form a complex with 1 and to phosphorylate in vitro serine 1152, a site also phosphorylated in vivo (Wang et al. 1996b). Phosphorylation of serine 1152 seems to be important for intracellular signaling mechanisms underlying L1-mediated neurite outgrowth, as a peptide containing this serine phosphorylation site will impair neurite outgrowth when introduced into chick dorsal root ganglion neurons.

The regulated tyrosine phosphorylation of L1 may have functional consequences, e.g., by mediating the recruitment of intracellular signaling proteins that bind tyrosine-phosphorylated motifs or influencing L1 interactions with cytoskeletal elements. The intracellular proteins that interact with L1 family proteins are largely unknown. Previous studies have linked cytoplasmic tyrosine kinases of the Src family, such as Fyn and Src, to the mechanism underlying L1-stimulated neurite outgrowth of cerebellar neurons (Ignelzi et al. 1994), and in principle, direct binding of these kinases through their Src homology 2 (SH2) domains to phosphorylated tyrosines in L1 is possible. The present evidence, however, favors an indirect association between L1 family proteins and Src family kinases. For example, the presence of Fyn in immunoprecipitates of the L1 family protein Ng-CAM from dorsal root ganglion neurons can be accounted for by its interaction with the GPI-anchored cell adhesion molecule axonin-1, which interacts with Ng-CAM (Kunz et al. 1996). L1 family proteins have also been reported to bind to the cytoskeletal protein ankyrin (Davis and Bennett 1994), but it is not known whether the phosphorylation state of L1 affects its mode of interaction with ankyrin.

The phosphorylation of L1 on tyrosine by EphB2 and on serine by $p90^{rsk}$ suggests that L1 signaling pathways exist that are not dependent on FGF receptor 1. Furthermore, L1 family proteins may only be the first among a number of adhesion molecules that will be found to engage Eph receptors for their function.

Beyond the surface: a two-hybrid approach to Eph receptor signaling

Studies of Eph receptor function have focused mainly on their role in axon guidance and fasciculation during pattern formation in the developing nervous system (reviewed by Drescher et al. 1997; Tessier-Lavigne and Goodman 1996), and more recently on their possible involvement in angiogenesis (Pandey et al. 1995b). The rapid identification of ligands for Eph receptors has led to new insights into the way in which cell-cell contact mediated interactions between Eph receptors and ephrins may contribute to the establishment of precise neuronal connections. The identification of the intracellular components of the signaling pathways that are activated by the interaction between Eph receptors and ephrins, however, lags far behind. Here, we summarize what is known about the signaling molecules that bind to activated Eph receptors.

The binding of a ligand to the extracellular domain of an Eph receptor tyrosine kinase results in autophosphorylation of its cytoplasmic domain and an increase in its catalytic activity. These events represent the initial steps that lead to activation of the signal transduction pathways of receptor tyrosine kinases (Van der Geer et al. 1994). Information about components that exert the responses downstream of Eph receptors is very limited. Efforts to elucidate the signaling pathways of Eph receptors have resulted in the notion that they are distinct from the known signaling pathways of other receptor tyrosine kinases, because activation of Eph receptors has demonstrated little or no effect on cell proliferation or cell transformation (Lhotak and Pawson 1993; Brambilla et al. 1995).

Five different proteins have been reported to interact with Eph receptors, phosphatidylinositol 3-kinase (PI 3-kinase; Pandey et al. 1994), Src-like adaptor protein (SLAP; Pandey et al. 1995a), the Src family kinase Fyn (Ellis et al. 1996), Grb2, and Grb10 (Stein et al. 1996). Four of them, PI 3-kinase, SLAP, Grb2, and Grb10, have been identified by using the yeast two-hybrid technique (Fields and Song 1989). With this technique, we have

shown that Src and PLCγ also interact with the Eph receptors EphA4 and EphB2 (unpublished). All the interactions identified with the two-hybrid system have been confirmed by biochemical assays. The molecules that have been identified belong to two categories. SLAP, Grb2, and Grb10 are adaptor molecules that lack enzymatic activity and presumably serve as a bridge to other unknown downstream signaling molecules. PI 3-kinase, PLCγ, and Src family tyrosine kinases possess intrinsic enzymatic activities that can directly mediate signal amplification. All of these signaling molecules possess SH2 domains, which serve as binding modules for specific tyrosine phosphorylated sequences in the cytoplasmic domains of the Eph receptors (Pawson and Schlessinger 1993). Thus, through their SH2 domains, the signaling molecules are able to bind to the activated receptors and propagate the signals.

PI 3-kinase has been identified as a binding partner for the EphA2 receptor (Pandey et al. 1994). The carboxy-terminal SH2 domain of the regulatory p85 subunit of PI 3-kinase binds to EphA2 immunoprecipitated from lysates of rat vascular smooth muscle cells treated with ephrin-A1 ligand. The activation of EphA2 by ephrin-A1 results in the stimulation of PI 3-kinase activity, which is known to generate D-3 phosphorylated phosphoinositides, a postulated second messenger that has been implicated in mediating cytoskeletal alterations (Janmey and Stossel 1989). A role for PI 3-kinase activity has been previously proposed in the signaling pathways of the PDGF receptor involved in membrane ruffling (Wennström et al. 1994) and proliferative responses (Valius and Kazlaukas 1993). The effects of PI 3-kinase on cytoskeletal architecture may also be of potential relevance in the nervous system, e.g., in the Eph receptor-mediated responses in the growth cone. The effect on proliferation presumably requires additional components that are not part of the signaling pathways of Eph receptors, which, as mentioned above, do not seem to mediate proliferative signals.

SLAP has been identified as a novel adaptor protein that binds to the phosphorylated cytoplasmic domain of the EphA2 receptor (Pandey et al. 1995a, b). SLAP binds activated EphA2 from rat vascular muscle cells via its SH2 domain, which exhibits 51% sequence identity to the SH2 domain of Src. SLAP, like other known adaptor proteins, such as Grb2, Crk, and Nck, also contains a Src homology 3 (SH3) domain. It is not known whether SLAP binds to Eph receptors other than EphA2, or to other families of receptor tyrosine kinases. It will be of interest to determine whether SLAP competes with Src family kinases for binding to the same tyrosine phosphorylated sequences.

Grb2 and Grb10 have been shown to bind the activated EphB1 receptor immunoprecipitated from vascular endothelial cells (Stein et al. 1996). Through its SH2 domain, Grb10 interacts with the phosphorylated tyrosine residue 929 in the carboxy-terminal domain of the EphB1 receptor. Grb2 also contains an SH2 domain but binds to another unknown phosphorylation site in the kinase domain of EphB1. Grb10 shares homology with the product of the gene locus F10E9.6 in *C. elegans*, which is crucial for the embryonic migration of a subset of *C. elegans* neuronal cells (Ooi et al. 1995 and references therein). Grb2 has been previously identified as an adaptor molecule in growth factor receptor signaling pathways leading to Ras activation and cell proliferation (Van der Geer et al. 1994). The specific roles of Grb2 and Grb10 in Eph-receptor-mediated signaling in vascular endothelial cells and in the nervous system remain to be elucidated.

The tissue distribution of Src family kinases and their cellular and subcellular localization suggest that they are promising candidates in Eph receptor signaling pathways in the nervous system. Several members of the Src family, including Src, Fyn and Yes, have expression patterns in the nervous system similar to those of Eph receptors. For example, in the chicken embryo, Src is highly concentrated in the molecular layer of the cerebellum and in the inner plexiform layer and nerve fiber layer of the retina (Sorge et al. 1984; Fults et al. 1985). Most notably, the Src kinases are expressed in developing neurons, where they are concentrated in axons and growth cones (Ingraham et al. 1992; Bixby and Jhabvala 1993). Recent studies indicate that the Src family kinase, Fyn, is able to bind the Eph receptor EphA4 (Gilardi-Hebenstreit et al. 1992). Fyn was identified by screening a number of SH2-domain-containing proteins for their ability to interact with a tyrosine phosphorylated glutathione S-transferase (GST) fusion protein of the cytoplasmic domain of EphA4 (Ellis et al. 1996). As shown by site-directed mutagenesis in conjunction with various in vitro binding assays, the stable interaction between EphA4 and Fyn is dependent upon autophosphorylation of tyrosine residue 602 within the sequence YEDP in the juxtamembrane region of mouse EphA4.

Studies in our laboratory now suggest that Src and Yes, in addition to Fyn, bind to Eph receptors (Zisch et al., unpublished). In vivo, phosphorylated EphA3, EphA4, and EphB2 from embryonic day-13 chicken neural retina all exhibit a high affinity for the SH2 domain of Src, but interstingly they vary in their relative affinities for the SH2 domains of other Src family kinases. In support of an in vivo interaction, we have found that Src coprecipitates with activated EphB2 from B28 rat glioblastoma cells transfected with the receptor. In the neuronal cell, a consequence of the interaction between Eph receptors and Src family kinases is presumably the recruitment of the Src kinases to sites at which activated Eph receptors are concentrated, namely axons and growth cones. In addition to being implicated in a variety of other signaling pathways (Superti-Furga and Courtneidge 1995), Src kinase activities modulated by the activation of Eph receptors may be partly responsible for the cytoskeletal alterations that occur upon cell-cell contact-mediated repulsion triggered by ligands for Eph receptors. Increased Src activity has been associated with changes in cytoskeletal organization (Parsons and Parsons 1997), such as those occurring during the migration of cells and growth cones.

Another signaling molecule interacting with Eph receptors has also been recently identified in a two-hybrid screen of a murine embryonic cDNA library by using the cytoplasmic domain of chicken EphA4 as a “bait”.

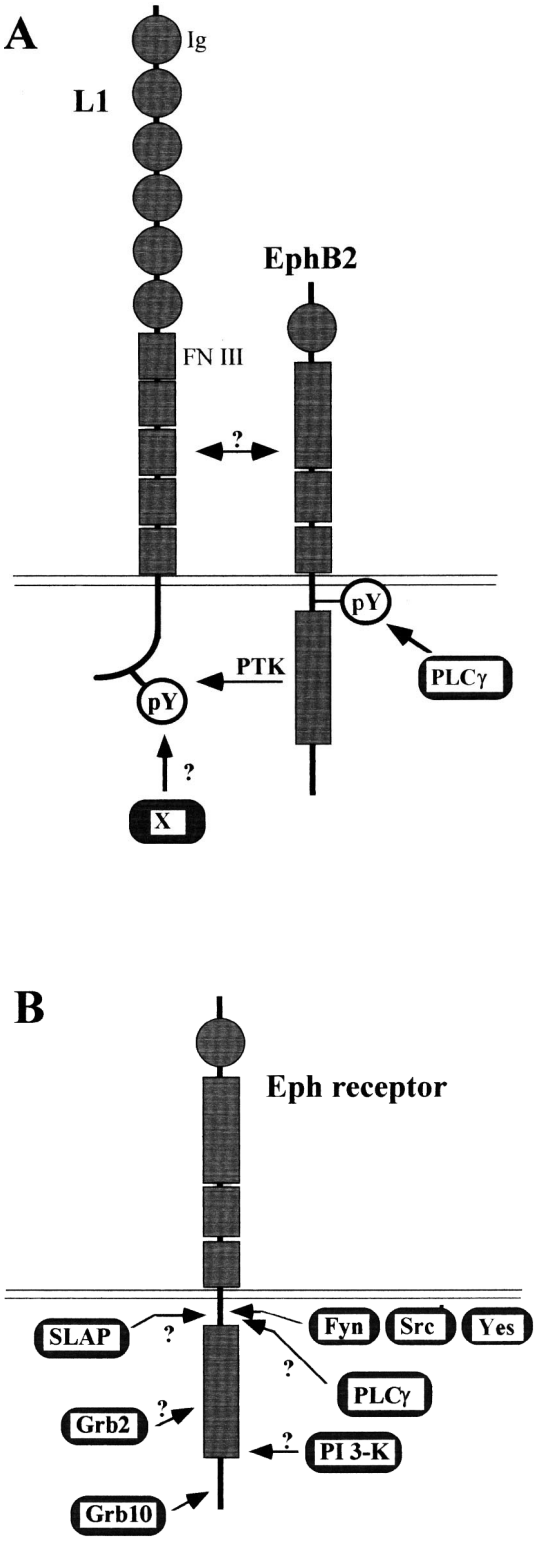

Fig. 2A, B. Eph receptor signaling. **A** Possible functional association between Eph receptors and neural cell adhesion molecules. Tyrosine phosphorylation of L1 protein by EphB2 may lead to conformational changes of the L1 intracellular domain and/or recruitment of as yet unknown cytoplasmic signaling molecules (*x*) to tyrosine phosphorylated acceptor sites (*pY*). *PTK*, protein tyrosine kinase. **B** Intracellular signaling interactions of Eph receptors. SH2-containing signaling proteins known to bind various Eph receptors are shown. *Arrows*, Demonstrated binding sites; *question marks*, unknown binding sites

In this screen, we obtained two independent clones comprising the carboxy-terminal SH2 domain of isoforms of PLCγ (unpublished). The interaction of EphA4 with PLCγ was confirmed by the co-immunoprecipitation of PLCγ and EphA4 from fibroblasts that were infected with Rous sarcoma virus, and that express elevated levels of tyrosine phosphorylated EphA4 (Zisch and Pasquale, unpublished). It has not, however, been determined whether activated Eph receptors associate with PLCγ in vivo in the nervous system.

We summarize the interactions of EphB2 with L1 family neuronal cell adhesion molecules, PLCγ, and other signaling molecules that may be effectors of Eph receptor signaling in neurons, in Fig. 2. Some features of this model are reminiscent of FGF receptor signaling in neurite outgrowth stimulated through neural cell adhesion molecules (Hall et al. 1996; Doherty and Walsh 1996). An intersting area of future investigation will be to characterize further the specific features of Eph receptor signaling that are crucial for their ability to guide neuronal projections to their targets.

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