EMBO MEMBER’S REVIEW

Ephrins and their Eph receptors: multitalented directors of embryonic development

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Keywords: angiogenesis/axon guidance/nervous system development/topography/tyrosine protein kinase

Introduction

Ephrins are membrane-bound ligands for the Eph family of protein tyrosine kinase receptors. Recent genetic studies have indicated that these molecules play key roles in diverse biological processes such as the development of the nervous system and angiogenesis. In the nervous system, they provide positional information by employing mechanisms that involve repulsion of migrating cells and growing axons. Understanding the mechanisms that mediate these biological responses will help to establish the molecular basis of topographic positioning within the developing embryo.

The molecules

Eph receptors have been conserved in a variety of eukaryotic species from Caenorhabditis elegans to man. They constitute the largest subgroup within the tyrosine kinase receptor family, with 14 receptors in mammals known to date. These receptors interact with cell-surface-bound ligands known as ephrins. The Eph receptors and their ephrin ligands can be divided into two classes based on structural features and binding affinities (Eph Nomenclature Committee, 1997; Figure 1). Type A ephrins are attached to the outer leaflet of the plasma membrane by a glycosyl phosphatidylinositol (GPI) moiety and they bind to the type A class of structurally related Eph receptors (Eph Nomenclature Committee, 1997). Type B ephrins have, in addition to their extracellular domain, a single transmembrane domain and a cytoplasmic tail. They bind to type B Eph receptors (Eph Nomenclature Committee, 1997). With the exception of EphA4, which can bind members of class A and class B ephrins, there appears to be very limited cross-talk between the A and B classes (Gale et al., 1996). Although there is a high degree of promiscuity between ephrins and Eph receptors of the same class, they may not be functionally interchangeable. For instance, there are considerable differences in binding affinities between different ligand–receptor pairs within the same class, suggesting that there may be preferred ligands for certain receptors (see e.g. Gale et al., 1996; Monschau et al., 1997).

All Eph receptors have an N-terminal globular domain which folds into a compact jellyroll β-sandwich (Himanen et al., 1998). This domain is necessary and sufficient for ligand binding (Labrador et al., 1997). The extracellular domain also contains two fibronectin type III domains, which serve to dimerize receptors (Lackmann et al., 1998), and two stretches of cysteine-rich sequence. The intracellular region has a single tyrosine protein kinase domain and a SAM domain. Eph receptors, as well as type B ephrins, have consensus sequences for binding PDZ proteins in the C-termini (Hock et al., 1998b; Torres et al., 1998; Lin et al., 1999).

Signaling pathways

Genes encoding transcription factors with a homeodomain, Hox genes, are key regulators in the patterning of the developing organism, but how these transcription factors mediate their effects remains unknown (Krumlauf, 1994). Evidence has started to accumulate suggesting that ephrins and Eph receptors function in the same genetic pathways as Hox genes and that, in some situations, they may be effectors of Hox genes. For example, the homeobox-containing protein Engrailed regulates the expression of axon guidance cues in the midbrain, and ephrins seem to be the main molecules directing the development of axonal projections in this system (Friedman and O’Leary, 1996; Itasaki and Nakamura, 1996; Logan et al., 1996). In line with this evidence, ectopic expression of Engrailed results in increased ephrin-A2 and ephrin-A5 expression in the chick midbrain (Logan et al., 1996; Shigetani et al., 1997). Furthermore, EphA7 expression is regulated by Hoxa2 as demonstrated by decreased EphA7 expression in Hoxa2−/− mice (Taneja et al., 1996), and Hoxa1 and Hoxb1 positively regulate EphA2 expression in vitro and in vivo (Chen and Ruley, 1998; Studer et al., 1998). In other situations the ephrin–Eph and Hox pathways may also act in parallel, since Krox-20, a regulator of Hox gene expression in the hindbrain, was recently found to directly regulate EphA4 expression in vivo (Theil et al., 1998).

The mechanisms by which these molecules mediate their downstream signaling are rapidly being unveiled (Brückner and Klein, 1998). Ephrins, which are monomers, appear to dimerize their receptors by forming aggregates in certain subdomains of the membrane. Soluble ephrins can bind their cognate Eph receptors, but they fail to activate them unless they are clustered (Davis et al., 1994). This clustering appears to be facilitated by their interaction with PDZ domain proteins (Hock et al., 1998b; Torres et al., 1998; Brückner et al., 1999; Lin et al., 1999). Interestingly, the degree of multimerization of the
EphB2 receptors have been found to be overexpressed in several different human tumors (Kiyokawa et al., 1994), yet there is no evidence that Eph receptors play a role in tumorigenesis. Indeed, Eph receptor activation fails to transform rodent fibroblasts in culture (Lhotak and Pawson, 1993). Thus, it is likely that these receptors may utilize, at least in part, signal transduction pathways distinct from those used by tyrosine kinase receptors involved in mitogenic signaling. Several proteins have been reported to bind to the intracellular domain of Eph receptors. These include p59fyn, PI3-kinase, Grb2, Grb10, RasGap, Nck, Crk and a novel kinase-less Src-like adaptor protein, SLAP (Pandey et al., 1994, 1995a; Ellis et al., 1996; Holland et al., 1997; Hock et al., 1998a). Ephrins induce rearrangements of the cytoskeleton in axonal growth cones (Meima et al., 1997a,b) and one pathway from the receptor to the cytoskeleton has been suggested to be initiated by the binding of RasGap to activated Eph receptors (Holland et al., 1997). The binding of RasGap induces the formation of a ternary complex containing p62\textsuperscript{dock} and Nck, where RasGap and Nck have been implicated in remodeling the cytoskeleton and axonal guidance (Holland et al., 1997).

Type B ephrins also signal in response to receptor binding, thus enabling bidirectional signaling. The first indication that transmembrane ephrins may have signaling capabilities came from genetic studies of mice carrying targeted EphB2 alleles. Henkemeyer et al. (1996) showed that whereas mice lacking EphB2 receptors had a malformed anterior commisure, similar mice expressing mutant receptors that lacked their kinase domains had no detectable defects. These results suggest that proper formation of the anterior commisure requires interaction between EphB2 receptors and their cognate B ephrins, but not signaling by these receptors. Indeed, binding of type B Eph receptors to transmembrane ephrins induces tyrosine phosphorylation in their cytoplasmic tails (Holland et al., 1996; Brückner et al., 1997). Since the type B ephrins lack endogenous kinase activity, it is presumed that they are phosphorylated by cytoplasmic kinases. Additional support for the view that Eph receptors may have functions that are independent of their kinase activity come from genetic studies in C. elegans as well as from the identification of EphB6, a kinase-dead Eph receptor (Gurniak and Berg, 1996; George et al., 1998). Finally, rapid tyrosine phosphorylation of ephrin-B1 upon exposure of cells to platelet-derived growth factor, and suppression of mitogenic properties mediated by tyrosine kinase receptors by co-expressed ephrin-B1, suggests cross-talk between other tyrosine kinase receptors and class B ephrins (Brambilla et al., 1996; Brückner et al., 1997).

**Cellular repulsion and formation of boundaries**

The first experiments that demonstrated the repulsive effect of an ephrin were the result of an ambitious search for repellent molecules in the chick visual system that were known to guide growing axons. In this study, ephrin-A5 was isolated and its repulsive effect demonstrated in \textit{in vitro} assays (Drescher et al., 1995). When retinal axons were allowed to choose between growing on ephrin-A5-containing or -depleted substrates in the stripe assay (Walter et al., 1987), they avoided ephrin-A5-containing lanes (Drescher et al., 1995). Ephrin-A2 was also found to repel retinal axons both \textit{in vitro} and \textit{in vivo} when ectopically expressed with a retroviral vector (Nakamoto et al., 1996). These initial experiments have been extended and there are now ample examples of axons from different neuronal types that are repelled by ephrins (Flanagan and Vanderhaegen, 1998).

The repulsion of axons induced by the interaction of ephrins with their cognate Eph receptors is believed to be mediated by rearrangements in the cytoskeleton of the axonal growth cone which result in retraction of the axon in response to a signal transduction event (Meima et al., 1997a,b). There is also increasing evidence that ephrins and their receptors may guide migrating cells by mediating a repulsive action and constraining cells to certain migratory routes. For example, migrating neural crest cells express Eph receptors, and ephrins are expressed in territories that the migrating cells normally avoid. Interference with ephrin–Eph interaction or signaling during...
neural crest migration disrupts the normal patterning of these cells (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997). In the branchial neural crest, ephrin-B2 is essential for restricting the intermingling of second- and third-arch neural crests, and for targeting third-arch neural crest cells to the correct destination (Smith et al., 1997). In the trunk, B-type ephrins expressed in the caudal half of the sclerotome direct the migration of neural crest cells through the rostral half (Krull et al., 1997; Wang and Anderson, 1997).

Ephrins and Eph receptors are expressed in gradients in some regions of the central nervous system, where they are implicated in the formation of topographic axonal projections (discussed below). In addition, they also appear in complementary and mutually exclusive domains, suggesting that these molecules may underlie boundary formation (Gale et al., 1996). Several lines of evidence suggest that axons and migrating cells sense differences in the concentration of ephrins, and that the graded expression or the sharp borders provide positional information. Misexpression of wild-type or dominant-negative forms of ephrins and Eph receptors in zebrafish embryos has proved to be a powerful approach in the study of the role of these genes in boundary formation. In the developing hindbrain, Eph receptors and type B ephrins in complementary segments (rhombomeres) restrict cell intermingling over boundaries (Xu et al., 1995, 1999). The sorting of cells to different domains appears to be dependent on bidirectional signaling (Mellitzer et al., 1999). However, unidirectional signaling through Eph receptors can restrict cell communication through gap junctions (Mellitzer et al., 1999). In addition to segmentation of the hindbrain, interruption of Eph signaling leads to abnormal somite formation, implicating Eph signaling in boundary formation and patterning of presomitic mesoderm into somites (Durbin et al., 1998).

Ephrins and Eph receptors also play an important role in establishing boundaries between arteries and veins during angiogenesis. For instance, in the initial stages of angiogenesis, presumptive arterial and venous endothelial cells can be distinguished by their selective expression of ephrin-B2 or an EphB receptor, respectively (Wang et al., 1998; Adams et al., 1999). In ephrin-B2-null mice, and in some EphB2/EphB3 double-deficient mice, angiogenesis is defective and the embryos die in mid-gestation (Wang et al., 1998; Adams et al., 1999). These findings indicate a novel role for ephrins and their receptors, and warrant further studies on how these molecules may participate in angiogenesis.

There are several examples in which the role of ephrins cannot be easily explained by repulsive action. For example, ephrin-A1 has chemoattractant effects on endothelial cells (Pandey et al., 1995b). Another study demonstrates that whereas axons from cortical neurons normally not projecting to ephrin-A5-containing cortical layers are repelled by ephrin-A5 in vitro, ephrin-A5 induces sprouting of axons that normally project to these layers (Castellani et al., 1998). Developmental defects in gene-targeted mice, such as the cleft palate observed in EphB2/EphB3 double-mutant mice (Orioli et al., 1996) and the cranial defects observed in ephrin-A5-null mice due to the failure of the neural folds to adhere in the dorsal midline (our unpublished data), are difficult to explain by postulating loss of ephrin-repulsive activity. These genetic studies suggest that ephrins and Eph receptors, either directly or indirectly, may have other biological activities, even cell adhesion in some contexts.

**Axon guidance**

Several studies in mutant mice lacking Eph receptors have illustrated the requirement for these molecules for correct axonal path finding in certain projections (summarized in Figure 2). EphA8 expression in the nervous system is restricted to small subpopulations of neurons in the superior colliculus, hindbrain and spinal cord (Park et al., 1997). In the superior colliculus, EphA8 is expressed in a gradient with the highest levels rostrally (Park et al., 1997). In EphA8-deficient mice, axons from a group of neurons in the superior colliculus (which normally express EphA8) fail to reach their normal target in the contralateral inferior colliculus, and instead extend into the ipsilateral...
Fig. 3. Topographic organization of retinal projections. (A) The retina projects in a topographic manner to the lateral geniculate nucleus in the forebrain and to the superior colliculus in the midbrain, allowing ordered transfer of information. (B) Depending on the location of a retinal ganglion cell in the retina on the dorso-ventral and naso-temporal axes, its axon will terminate at a distinct position along the antero-posterior and medio-lateral axes, respectively, of the superior colliculus. (C) Three scenarios where neurons project to a target area indicated by the broken line. In the upper panel, all neurons are equally sensitive to the graded ligand and will reach the threshold where the ligand induces them to terminate at the same point. In the middle panel, the neurons are differentially sensitive to the ligand, but since the ligand is present in a uniform concentration, an axon will either terminate upon reaching the target or grow through it if the concentration is too low to induce the axon to stop. In the lower panel, both receptor and ligand are present in gradients, allowing the establishment of a topographic map in which individual axons terminate at distinct positions within the target.

cervical spinal cord (Park et al., 1997). EphA4-null mice exhibit motor dysfunction, which is probably caused by disruption of the corticospinal tract (Dottori et al., 1998). In addition, in the majority of these mice, the anterior commissure is missing (Dottori et al., 1998).

EphB3-null mice display a partially penetrant defect in the formation of the corpus callosum, where in some animals the axons fail to cross the midline and instead form bundles of axons at the midline or grow along the antero-posterior axis instead of crossing the midline (Orioli et al., 1996). In mice lacking EphB2 receptors, the posterior part of the anterior commissure is malformed with many of the axons that normally form this tract projecting aberrantly to the floor of the brain (Henkemeyer et al., 1996). Interestingly, the axons of the anterior commissure do not express EphB2 receptors, but express the EphB2 ligand ephrin-B1, whereas EphB2 is expressed in cells surrounding the growing axons (Henkemeyer et al., 1996). This lends strong support to the concept that B ephrins may signal upon Eph receptor binding. Finally, EphB2 and EphB3 double-mutant mice show higher penetrance of the partial defects seen in mice in which only one gene has been deleted (Orioli et al., 1996), demonstrating limited functional redundancy between different Eph receptors.

These double-mutant mice also show defective fasciculation of axons in the habenular–interpeduncular tract, although these axons reach their target (Orioli et al., 1996). These results suggest a role for ephrins and Eph receptors in axon fasciculation independent of axon guidance. Previous in vitro studies have demonstrated that blocking ephrin–Eph-receptor interactions in co-cultures of cortical neurons and astrocytes with soluble chimeric ligand or receptor molecules results in defasciculated axon growth (Winslow et al., 1995). How ephrins and Eph receptors might participate in axonal fasciculation is not clear. They may mediate cell adhesion or induce the synthesis of cell adhesion molecules. Alternatively, Eph-receptor-expressing neurons may favor growing on each other rather than on astrocytes expressing ephrins, resulting in fascicle formation (Tessier-Lavigne, 1995).

**Formation of topographic axonal projections**

In 1963, Sperry suggested that gradients of a few molecules may guide growing axons to distinct locations in a topographic projection. This theory requires that neurons are differentially sensitive to such molecules, a situation that might be achieved by graded expression of their receptors (Figure 3). The identification of graded expression of ephrins, along with their repulsive activity, has lent support to the hypothesis that these molecules may play a role in the formation of topographic maps. The pre-eminent model system for studies on topographic projections has been the visual system, where retinal ganglion cells project in a topographic manner to several targets, mainly the superior colliculus (tectum in birds) in the midbrain and the lateral geniculate nucleus in the forebrain. The projection of retinal axons is topographically ordered along two axes. Depending on the location of a given neuron on the dorso-ventral and naso-temporal axes of the retina, its axon will terminate at distinct positions along the medio-lateral and antero-posterior axes.
of the superior colliculus (Figure 3B) and the lateral geniculate nucleus, respectively.

Individual retinal ganglion cells express different levels of type A Eph receptors along the naso-temporal axis, creating a smooth gradient of receptor expression and type A ephrin sensitivity (Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996; Monschau et al., 1997; Feldheim et al., 1998). Two closely related ephrins, ephrin-A2 and ephrin-A5, are expressed in overlapping gradients in both the superior colliculus and lateral geniculate nucleus (Cheng et al., 1995; Drescher et al., 1995; Feldheim et al., 1998). Altering the smooth gradient of ephrin expression by retroviral misexpression in the chick tectum induces axons to terminate at patches of high ephrin expression and disrupts the normal topography of this projection, providing the first in vivo evidence for a role of ephrins in topographic mapping (Nakamoto et al., 1996).

Genetic studies have provided additional evidence for the role of ephrins and their cognate Eph receptors in topographic mapping. Analysis of retinal projections in ephrin-A5-null mice has demonstrated two distinct functions for ephrin-A5 in the projection of retinal ganglion cell axons to their targets at two different developmental phases (Frisén et al., 1998b). In neonatal wild-type and ephrin-A5-null mice, most retinal ganglion cell axons have reached the superior colliculus. However, in ephrin-A5-null animals, many axons extend beyond the superior colliculus into the inferior colliculus (Frisén et al., 1998b). This overshooting suggests that ephrin-A5, which is highly expressed in the inferior colliculus, serves as a stop signal for axons to terminate within its target. Ephrin-A5 plays its second role in the formation of the retino-collicular projection during the phase of establishment of topography. In ephrin-A5-null mice, a substantial number of axons terminate at topographically incorrect locations (Frisén et al., 1998b). Axons from temporal neurons, which normally project to the anterior superior colliculus, often terminate at more posterior regions.

Ephrin-A5 also serves as a topographic cue for retinal projections to the lateral geniculate nucleus (Feldheim et al., 1998). Interestingly, the projection of both temporal and nasal retinal neurons to the lateral geniculate nucleus is affected in the ephrin-A5-null mice, lending strong support to the hypothesis that axons compete for space relative to one another, not relative to the target (Feldheim et al., 1998). The use of the same set of molecules to establish topography in different targets for retinal neurons bears resemblance to the metameric organization of the body along the anterior–posterior axis and has implications for the emergence of new targets in the central nervous system during evolution (Feldheim et al., 1998).

In addition to the expression of Eph receptors in the retina, several ligands are expressed in the retina, and some of them in a gradient. Modulation of type A ephrin levels in retinal neurons in vitro and in vivo results in altered sensitivity of the axons of these neurons to ephrins (Hornberger et al., 1999). This has led to the suggestion that the responsiveness of a neuron to an ephrin is regulated not only by the level of receptor expression but also by co-expression of ligand, which may render the neuron less sensitive to ephrin (Hornberger et al., 1999).

Although the role of ephrins in directing the development of topographic projections has been tested only in the visual system, there are reasons to believe that they may serve similar roles in other projections. For instance, the expression patterns of ephrins and Eph receptors in the thalamo-cortical projections, septo-hippocampal tract, nigro-striatal pathway and motor neuron projections to muscles along the anterior–posterior axis implicate these molecules in the organization of the topography of these systems (Donoghue et al., 1996; Gao et al., 1996, 1998a; Yue et al., 1999).

Is there a role for ephrins and Eph receptors beyond embryonic development?

In spite of the large number of studies describing important functions for ephrins and Eph receptors during embryonic development, there are very few that address the role of these molecules in the adult. Most ephrins and Eph receptors are predominantly expressed during development, but several are also expressed in adult tissues. Based on the actions of these genes during development, one may suspect that these molecules could act in plasticity processes. Indeed, in the adult brain, the expression of several ephrins and Eph receptors is most prominent in plastic regions. For example, Eph receptors are found in synapses of the adult hippocampus (Buchert et al., 1999). Interfering with Eph signaling by injection of blocking or activating agents has indicated a role for ephrins in synaptic remodeling and long-term potentiation (Gao et al., 1998b). Moreover, it is also possible that ephrins and Eph receptors may take part in guiding migration and connectivity of new neurons generated from stem cells in the adult brain (Frisén et al., 1998a). Outside the nervous system, it is tempting to speculate that ephrins and their Eph receptors may also play a role in adult angiogenesis.

Acknowledgements

Work in the authors’ laboratory described in this review is supported by the Swedish Medical Research Council, the Swedish Cancer Society and the Swedish Foundation for Strategic Research to J.F. M.B. is supported by grants from Pfizer Inc. and the Plan Nacional de I+D from the Ministerio de Educación y Ciencia of Spain.

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Received March 18, 1999; revised and accepted August 6, 1999.