Eph receptors and ephrins: effectors of morphogenesis

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SUMMARY

Eph receptor tyrosine kinases and their ligands, the ephrins, appear to lie functionally at the interface between pattern formation and morphogenesis. We review the role of Eph and ephrin signalling in the formation of segmented structures, in the control of axon guidance and cell migration and in the development of the vasculature. We address the question of how the specificity of response is achieved and discuss the specificity of ephrin-Eph interactions and the significance of structural domains in Eph receptors.

Key words: Eph receptors, Ephrins, Morphogenesis

(A) INTRODUCTION

The creation of form in the embryo requires the coordinated behaviour of cells. From the original collection of blastomeres, either in groups or as individuals, cells undergo choreographed changes in cell shape leading to movements and the organisation of cellular layers and boundaries. These cell sheets in turn bend and fold to generate tissues and organs. The cell behaviours that underlie morphogenesis occur as a result of alterations to the cytoskeleton, to the control of cell division and to the regulation of gene transcription. These events are stimulated as a result of prompts from the cells environment, generated as a result of the patterning processes at work in the early embryo. Many of the genes important for patterning the early embryo are now identified but the interface between patterning and morphogenesis remains unclear. This interface is fundamental to our understanding of how development works because it integrates the two processes of patterning and morphogenesis. In this review, we survey the biology of a family of receptor tyrosine kinases, the Eph receptor proteins and their ligands, the ephrins, which appear to lie functionally at the interface between pattern formation and the generation of form. Both Eph receptors and ephrins are dynamically expressed in a wide range of regions of the vertebrate embryo, in the ectoderm, mesoderm and endoderm and experimental evidence shows that they are required for formation of correct migration of cells or their processes, for the formation of boundaries between structures and for the control of cell shape.

We review the role of Eph receptor and ephrin signalling in the formation of rhombomeres and somites where reciprocal regions of expression of receptor and ligand in adjacent groups of cells result in interactions controlling cell behaviour at the interface. Evidence that Eph/ephrin signalling is involved in the control of cell movement comes from experiments involving such processes as guidance of axonal growth cones during the creation of the nervous system and migration of neural crest cells (Flanagan and Vanderhaeghen, 1998). Much of the evidence that is considered points to Eph receptor signalling leading to regulation of the cytoskeleton.

Receptor tyrosine kinases (RTKs) are membrane spanning proteins with an extracellular ligand-binding domain and an intracellular kinase domain. There are at least fourteen subfamilies of RTKs (Van der Geer et al., 1994) and the Eph subfamily is the largest (Orioli and Klein, 1997; Pasquale, 1997; Tuzi and Gullick, 1994). Each subfamily has characteristic ligand-binding and kinase domains and is activated by a distinct ligand or group of ligands. Many RTKs play roles in a broad range of processes in development.

In 1987, Hirai and co-workers described the cloning and characterisation of the first member of the Eph subfamily, EphA1 (Hirai et al., 1987). To date as many as fourteen genes have been described that are related to Eph by sequence and by general characteristics of their kinase and extracellular domains. Members of this subfamily of receptors have been isolated and characterised in a range of vertebrate species, including human, mouse, rat, chicken, Xenopus and zebrafish. They have also been isolated in invertebrates (George et al., 1998), but the invertebrate genes have been studied much less

*Editors note: This article was submitted in December 1998, shortly before the death of Nigel Holder, revised by Rüdiger Klein, and accepted for publication in Development in February 1999. Nigel will be sadly missed by his many friends and colleagues in the community of developmental biologists. He was an outstanding scientist and true friend.
The formation of cell processes is fundamental to the movement and morphogenesis of cells. Two well-studied examples of cell process formation and cell movement in embryos are growth cone extension from differentiating neurons and neural crest cell migration. We outline below the evidence that Eph/ephrin signalling is an important component of the mechanism underlying both events.

(B) FUNCTION OF EPH SIGNALLING IN EMBRYOGENESIS

(1) Segmentation

Segmentation is a basic process in embryogenesis of many invertebrate and all vertebrate embryos. In vertebrates, the two regions of the body axis that are clearly segmented are the paraxial mesoderm, which gives rise to the somites – the precursors of the segmented vertebral column (Gossler and Hrabé de Angelis, 1998), and the hindbrain region of the neural plate. The hindbrain is divided up into regular units called rhombomeres, which are the basis for patterning of the neural epithelium and subsequent differentiation of neurons (Lumsden and Krumlauf, 1996). In both regions of the embryo, the segments develop clear boundaries at which the cells undergo distinctive behaviours involving cell shape changes. In the hindbrain, segmentation occurs within a defined region of the neural plate whereas, in the paraxial mesoderm, segmentation is a dynamic process linked to the growth of the body axis at the posterior end. Eph receptors and ephrins express in both hindbrain neural plate and paraxial mesoderm and functional analysis of receptor signalling in zebrafish and in *Xenopus* indicates that such signalling is crucial for normal development of segment boundaries in both regions of the embryo (Fig. 1).

The vertebrate hindbrain consists of 7 or 8 rhombomeres, which become apparent during the neurulation stages, once gastrulation is completed. Boundaries develop gradually and in a predictable sequence. The boundaries are evident because cells within the boundary zone have specific flattened shapes and are organised in straight lines at right angles to the body axis (Heyman et al., 1993; Moens et al., 1998). These edges are boundaries to cell movement and are extremes for expression of genes concerned with patterning the hindbrain (Lumsden and Krølhaug, 1996). Eph receptors and ephrins are expressed in specific rhombomeres such that receptors and ligands interact at the future boundaries. For example, in *Xenopus* and other vertebrates EphA4 is expressed in rhombomere 3 and 5 (Becker et al., 1994; Gale et al., 1996; Xu et al., 1995) and *Xenopus* and mouse ephrinB2 is expressed in rhombomeres 2, 4 and 6 (Bergmann et al., 1995; Smith et al., 1997). The fields of cells in these alternating rhombomeres interact only at the future boundaries between the rhombomeres. This interpretation is consistent with grafting experiments in the chick embryo in which it has been shown that interfaces between alternating rhombomeres are necessary...
domains form alternating rhombomeres but, in the somites, as compared to that in the hindbrain. In the latter, expression arrangement of Eph receptor/ephrin expression in the somites is misplaced or absent in experimental embryos (Fig. 1). As in the hindbrain rhombomeres, somite boundaries are required for normal somite segmentation (Durbin et al., 1998). It has been shown that Eph receptor/ephrin signalling is inactive receptors or soluble ephrins into the zebrafish embryo, using a dominant negative strategy injecting RNA encoding kinase (Bergemann et al., 1995; Cooke et al., 1997; Flenniken et al., 1996). EphA4 expression in rhombomeres 3 and 5 of the paraxial mesoderm is that a somite segment border forms at every other interface between anterior and posterior regions of the somite are required for maintenance of somite boundaries (Stern and Keynes, 1987). One feature of the presomitic expression of Eph receptors and ephrins in the paraxial mesoderm is that a somite segment border forms at every other interface between receptor- and ligand-expressing cells. It remains unclear why alternating interfaces between receptor field and ligand field should behave differently but it suggests that additional molecules are important in the process of somite segmentation.

Recent results in the zebrafish embryo show that a similar process, based on the expression of alternating stripes of Eph receptors and ephrins is important for normal somite segmentation to occur. Several Eph receptors and ephrins are expressed in the somitic mesoderm in a number of vertebrate species (Bergemann et al., 1995; Cooke et al., 1997; Flenniken et al., 1996; Gale et al., 1996; Scales et al., 1995). Using a dominant negative strategy injecting RNA encoding kinase inactive receptors or soluble ephrins into the zebrafish embryo, it has been shown that Eph receptor/ephrin signalling is required for normal somite segmentation (Durbin et al., 1998). As in the hindbrain rhombomeres, somite boundaries are misplaced or absent in experimental embryos (Fig. 1).

There is an important difference between the spatial arrangement of Eph receptor/ephrin expression in the somites as compared to that in the hindbrain. In the latter, expression domains form alternating rhombomeres but, in the somites, expression domains of Eph receptor and ephrin form anterior and posterior halves to a single somite. This is consistent with grafting experiments in the chick embryo in which it was shown that interfaces between anterior and posterior regions of the somite are required for maintenance of somite boundaries (Stern and Keynes, 1987). One feature of the presomitic expression of Eph receptors and ephrins in the paraxial mesoderm is that a somite segment border forms at every other interface between receptor- and ligand-expressing cells. It remains unclear why alternating interfaces between receptor field and ligand field should behave differently but it suggests that additional molecules are important in the process of somite segmentation.

It is of considerable interest to know how these expression domains are controlled in the forming hindbrain and in the paraxial mesoderm. In the hindbrain, it is known that the EphA4 expression in rhombomeres 3 and 5 is under the control of the transcriptional regulator Krox-20 (Irving et al., 1996; Theil et al., 1998). It is not yet clear how the dynamic expression of EphA4 and ephrinB2 is controlled in the unsegmented somitic mesoderm. Interactions at alternating interfaces between anterior and posterior half segments leads to boundary formation (arrows). Based on Durbin et al. (1998).
except for the location of the eye stalk. EphA4 expression persists in the ventral and dorsal diencephalic regions. In embryos injected with the dominant-negative RNA, the forebrain regions fated to become ventral diencephalon become retina instead and large expanded eyes are formed. Again, the exact role of EphA4 in the regionalisation process is unclear; however, since extensive morphogenetic movements underlie eye and ventral diencephalic tissue development, an involvement of EphA4 in cell association or the formation of a boundary is possible.

(2) Axon guidance and fasciculation

A vital aspect of the neuronal differentiation process is the production of an axon that grows and seeks a specific synaptic partner. It is evident from work on the retinotectal system (see below) that Eph signalling is involved in guiding axon growth. Initial evidence for such a role in the formation of centrally and peripherally projecting axon tracts arose from studies of the EphB2 receptor (Pasquale et al., 1992). EphB2 is expressed in a number of domains in the chick and mouse brain, including the retina. It has been demonstrated that EphB2 in the chick retina is highly phosphorylated, particularly during the phase when interneuronal contacts are established (Pasquale et al., 1994). To further implicate EphB2 function in the formation of appropriate neuronal contacts, this receptor has been immunolocalised to the surface of growth cones of spinal motor neuron and occulomotor neuron axons from the onset of their growth towards their respective targets (Henkemeyer et al., 1994). Furthermore, functional studies using a different receptor, EphA5, and a ligand by which it is activated, ephrinA5, showed directly that Eph receptor signalling is involved in axon fasciculation (Winslow et al., 1995). In these experiments, fasciculation of axons from cortical neurons growing on astrocytes was inhibited by soluble forms of both the receptor and the ligand, which are assumed to act in a dominant-negative manner. The strongest evidence to date implicating EphB2 in axon guidance in the embryo comes from targeted mutation studies in the mouse (Henkemeyer et al., 1996). An embryo homozygous for loss of EphB2 function lacks part of the anterior commissure. However, as the neurons projecting axons across the anterior commissure do not express EphB2, the authors suggest that the phenotype may reflect loss of signalling through the ligand to which it normally binds. Loss of commissural axons is more dramatic in mice null for both EphB2 and EphB3 (Orioli et al., 1996). In such animals, the anterior commissure and the corpus callosum are affected as well as the forming palate, an area of the embryo in which both receptors are normally expressed. It was further observed that at least one CNS axon bundle running in the anterior-posterior direction, the habenular-interpeduncle tract, was partially defasciculated, although the projection to its target, the ventral midbrain, appeared normal.

Eph receptors and ephrins are important for the formation of topographic maps in the visual system

The identification of two ephrins showing graded distribution from posterior to anterior in the developing chick midbrain tectum suggested a role for Eph signalling in establishing appropriate connections in the retinotectal system. An Eph ligand, ephrinA5, was purified from the chick tectum as a result of its expression in the tectum and the fact that it is a GPI-linked protein (Drescher et al., 1995). This was the culmination of a long and elegant series of experiments from Friedrich Bonhoeffer’s laboratory in which bioassays were devised that characterised the growth of chick retinal axons over the tectum. These experiments showed that membranes isolated from tectal cells possessed a collapsing activity for growth cones from temporal but not nasal retinal ganglion cells. EphrinA5, a GPI-linked family member, mimicked this collapsing activity. The existence of a second ligand, ephrinA2, was shown in the midbrain by binding of a chimeric protein in which the extracellular domain of a receptor was linked to alkaline phosphatase. The ephrin A2 cDNA was then expression cloned (Cheng and Flanagan, 1994).

In the chick, the ephrinA5 and ephrinA2 ligands are assumed to interact with two Eph receptors, EphA4, which is expressed uniformly across the retina, and EphA3, whose expression is graded across the chick retina, with a high point in the temporal region (Cheng et al., 1995). Interestingly, in the mouse, EphA3 is not prominently expressed in the ganglion cell layer, while EphA5 expression is found in a low nasal to high temporal gradient (Feldheim et al., 1998). In the chick tectum, the expression domains of the ligands differ from each other, with ephrinA2 extending more anteriorly than ephrinA5. With variations in ligand-binding specificities and the graded distributions of the ligands and of EphA3 and EphA4 (Monschau et al., 1997), it is feasible that sufficient information can be provided by Eph signalling to resolve the retinotopic map. In addition, Eph receptors and ligands are spatially regulated with respect to the dorsal-ventral axis of the eye. For instance, EphB2 is expressed more strongly in the ventral than dorsal retina (Holash and Pasquale, 1995; Kenny et al., 1995). Similarly, ephrins of the A class exist in a high nasal low temporal gradient and ephrins of the B class are expressed higher dorsally than ventrally (Marcus et al., 1996). Such localisation of ligands in the eye also occurs in the zebrafish where three ephrins are differentially expressed in retinal ganglion cells prior to and during the projection of axons to the midbrain (Brennan et al., 1997).

Recently, direct evidence has been provided that Eph signalling is involved in map formation by misexpressing ephrinA2 in the developing chick tectum and by showing that this leads to abnormal retinotectal axon growth (Nakamoto et al., 1996). Furthermore, a mouse carrying a mutation in the ephrinA5 gene has abnormal projections of retinal ganglion cells to the tectum. Although the gross aspects of the topographic map are normal in these mutants, axons normally projecting to the caudal end grow further into the hindbrain region than they would normally (Frisen et al., 1998). This result indicates that ephrinA5 acts primarily as block to axon growth. It is, however, not clear that all vertebrates will pattern information can be provided by Eph signalling to resolve the retinotopic map. In addition, Eph receptors and ligands are spatially regulated with respect to the dorsal-ventral axis of the eye. For instance, EphB2 is expressed more strongly in the ventral than dorsal retina (Holash and Pasquale, 1995; Kenny et al., 1995). Similarly, ephrins of the A class exist in a high nasal low temporal gradient and ephrins of the B class are expressed higher dorsally than ventrally (Marcus et al., 1996). Such localisation of ligands in the eye also occurs in the zebrafish where three ephrins are differentially expressed in retinal ganglion cells prior to and during the projection of axons to the midbrain (Brennan et al., 1997).

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Targeted mutation of the mouse EphA4 gene is absent from the eye (Xu et al., 1996). Targeted mutation of the mouse EphA4 homologue is absent from the eye (Xu et al., 1996). Targeted mutation of the mouse EphA4 gene, which is normally expressed in a rostrocaudal gradient in the eye and in the superior colliculus, has shown a requirement for EphA8 in the formation of normal contralateral connections between the superior colliculi as well as connections from the superior colliculus to the spinal cord (Park et al., 1997). However, the
nature of these EphA8-dependent projections has not yet been determined, i.e. whether they are involved in visual function or have other roles.

The challenge of understanding the role of Eph/ephrin signalling in the retinotectal system relates to the manner in which the growth cone of the retinal ganglion cell axon interprets a target field across which the cells express graded amounts of ephrins. It is not as yet clear how a growth cone expressing a particular amount of receptor responds to a graded signal. A clue may come from recent evidence in which differential Eph receptor signalling responses can be measured depending on the oligomeric properties of the ligand (Stein et al., 1998) (see below).

In contrast to the midbrain, topographic mapping in the forebrain is much less understood. Recent work from the Flanagan laboratory indicates that ephrinA2 and ephrinA5 ligands are topographic guidance molecules in the thalamic dorsal lateral geniculate nucleus (dLGN), a major relay station for retinal inputs to the visual cortex (Feldheim et al., 1998). Both ephrinA2 and ephrinA5 are expressed in gradients in the mouse dLGN and loss-of-function experiments show that ephrinA5 is necessary for correct topographic development of the dLGN map. These experiments nicely show that the same set of labels are used repeatedly in different targets for the same field of projecting neurons.

Eph signalling may be important for creating patterned neural connections elsewhere in the central and peripheral nervous system

The formation of topographic maps is not limited to the retinotectal system and is a feature of other regions of the CNS such as the hippocampus and septum which are areas involved in learning and memory. In this case, it has recently been shown that an Eph signalling system may underlie the formation of topographic projections involving the septum and hippocampus (Gao et al., 1996; Zhang et al., 1996). The hippocampus projects to the lateral septum in a precise order and receives input from the medial septum. EphrinA2 is expressed in a gradient from dorsal to ventral septum and, in culture, selectively allows growth of axons from appropriate regions of the hippocampus. In addition, the receptor EphA5 is expressed in a complementary lateral-to-medial gradient in the hippocampus.

Expression pattern data suggest that Eph receptor signalling may be involved in establishing specific neuronal connections elsewhere in the developing nervous system. For example, the mouse receptor EphA3, and its rat and chick homologues, are expressed in a subset of spinal motor neurons and a subset of axial muscles (Kilpatrick et al., 1996, Ohta et al., 1996). Furthermore, in the mouse, the ligand ephrinA5 is expressed to a greater extent by head and neck than by trunk and limb muscles and muscle cell lines derived from these different axial levels inhibit growth of dorsal root ganglion axons to different extents (Donoghue et al., 1996). These results suggest that specificity in connections of spinal motor and ganglionic neurons to the periphery may be based on Eph signalling.

Eph receptor/ephrin signalling leads to growth cone collapse

A number of studies have now shown that Eph receptor/ephrin signalling leads to collapse of the neuronal growth cone, leading to axon guidance by inhibition. Using the stripe and growth cone collapse assays developed by Friedrich Bonhoeffer’s laboratory, it has been shown that the class A ephrins expressed in the superior colliculus or tectum of mouse, chick and zebrafish cause growth cone collapse or repulsion in such assays (Brennan et al., 1997; Drescher et al., 1995; Nakamoto et al., 1996). This is also the case for chick spinal motor neurons, which express EphA4 and EphB2 and collapse following interactions with ephrins of the A and B class in in vitro assays (Henkemeyer et al., 1994; Ohta et al., 1997; Wang and Anderson, 1997).

Observations of growth cone activity in cultured CNS neurons following interactions with ephrins show that they collapse by withdrawal of filopodia and this has focused attention on the link between Eph receptor and class B ephrin signalling on the elements of the cytoskeleton. Evidence from in vitro assays, such as that outlined above, and from targeted mutation studies, such as that with EphB2 (Henkemeyer et al., 1996), indicate that signalling via class A or B receptors or via class B ephrins can lead to abnormal growth cone guidance. Recent work with cortical neurons in culture suggests that their responses to class A versus class B ephrins may be different in terms of the cytoskeletal components involved (Meima et al., 1997a,b). Interaction with ephrinA5 leads to alterations in actin polymerisation in cortical growth cones whereas ephrinB1 does not cause actin rearrangement but appears to affect microtubules in the growth cone.

(3) Eph signalling is involved in controlling cell migration

Gastrulation is the period of development when cells first begin to migrate during development. Two studies, both in Xenopus, indicate that interrupting normal signalling of EphA4 and the likely homologue of ephrinB1 can lead to abnormal adhesion of blastomeres during gastrulation (Jones et al., 1998; Winning et al., 1996). Overexpression of dominant negative forms of both receptor or ephrin causes the blastomeres to dissociate and gastrulation to abort. It is not likely that these effects are due to adhesive properties of the Eph receptor/ephrins themselves because adhesion is interrupted by overexpressing forms of ephrinB1 that lack the extracellular domain (Jones et al., 1998). Furthermore, these authors showed that the lack of adhesion between blastomeres can be rescued by overexpressing a cadherin at the same time as the truncated ephrin. However, it was not possible to show a direct link between ephrinB1 signalling and cadherin signalling in this situation. A potential role for Eph receptor/ephrin signalling during gastrulation is supported by the observation that several receptors and ephrins are dynamically expressed during zebrafish gastrulation (Cooke et al., 1997; Durbin et al., 1998; Xu et al., 1994).

Recent data from two independent lines of investigation have demonstrated a role for Eph signalling in controlling cell migration in embryos. The first involves the migration of neural crest (Fig. 2). In the trunk and the head neural crest, cells take particular paths to reach the regions of the periphery in which they will settle and differentiate. In the trunk of the rat and chick embryo, for example, crest cells are excluded from migrating through the caudal half somite. Two studies have now shown that this pattern of crest cell migration is in part
due to an inhibition of crest cell movement mediated by an Eph receptor, shown to be EphB3 in the chick, which is expressed on crest cells and cells of the rostral half somite and ephrinB1, which is expressed in the caudal somite (Krull et al., 1997; Wang and Anderson, 1997). In the rat, the receptor involved in this process is EphB2 and the ephrin expressed in the caudal half somite is ephrinB2. Therefore, different classes of class B ligands and receptors perform the task in different species.

A similar process of spatial exclusion underlies directed crest cell movement in the hindbrain where cells migrate from specific rhombomere regions to specific branchial arches (Smith et al., 1997). This study concentrated on the migration streams of neural crest from rhombomeres 4, 5 and 6, which distribute crest cells to branchial arches 2, 3 and 4, respectively (Fig. 2). Expression of ephrinB2 by crest cells from R4 prevents them from mixing with crest cells migrating from R5, which express EphA4 and EphB1 receptors, which can both be activated following binding to ephrinB2.

The second line of investigation involves a genetic analysis of the VAB-1 locus in C. elegans, which encodes an Eph receptor (George et al., 1998). In worms carrying null mutations in VAB-1, two processes, both involving cell movements, are affected following gastrulation. These processes are the movement of neuroblasts during closure of the ventral gastrulation cleft and migration of epidermal cells during ventral enclosure of the epidermis. Analysis of the phenotype of a group of mutant alleles shows that strong phenotypes involve mutations in the extracellular domain of the protein and weak phenotypes involve mutations in the intracellular kinase domain. These results suggest that VAB-1 may participate in forward and reverse signalling during the execution of these two morphogenetic events. The C. elegans embryo may provide valuable insights into the function of Eph/ephrin signalling in a system with fewer family members to consider.

(4) Ephrin/Eph function in development of the vascular system

EphrinA1 was originally isolated from a screen in which differential hybridisation was used to identify immediate-early response genes following cytokine stimulation of human umbilical vein endothelial cells (HUVECs) (Holzman et al., 1990). EphrinA1-Ig chimeric protein stimulates neovascularisation of the cornea. It has subsequently been shown that ephrinA1 binds to the EphA2 receptor present on the HUVECs and is responsible for controlling migration but not proliferation of these cells (Pandey et al., 1995c). The expression of the ligand is induced by TNF-α and, given that receptor activation requires ligands to be membrane bound (Davis et al., 1994), the ligand can act by activating receptor only on cells in its immediate environment.

During embryogenesis, blood vessel formation occurs in two distinct processes. Vasculogenesis defines the formation of a primary capillary network by fusion of endothelial precursor cells. It includes the in situ generation of the primordia of the heart and major trunk vessels, such as dorsal aorta and cardinal veins. In a second angiogenic process, the primary network is remodelled into a hierarchical set of large and small vessels and avascular tissues are vascularized by sprouting of new capillaries from existing vessels. EphrinB2-deficient mice suffer from severe disruption of the embryonic vasculature due to lack of remodeling of the primary capillary network (Wang et al., 1998). EphrinB2 is exclusively expressed in embryonic arteries, while one of its cognate receptors, EphB4, shows complementary expression in veins. It was suggested that reciprocal, possibly repulsive, signalling between these two types of vessels is required for remodelling of the embryonic vasculature (Wang et al., 1998). Our own work (R. K.) subsequently showed that other members of the B class of ephrins (ephrinB1) and Eph receptors (EphB2 and EphB3) are either co-expressed on endothelial cells, or at endothelial-mesenchymal cell boundaries (Adams et al., 1999). Consistent with these expression patterns, double mutant EphB2/EphB3-deficient mice have a partially penetrant phenotype that resembles the ephrinB2 knockout phenotype. These findings indicate that ephrin/Eph signalling occurs and is required throughout the embryonic vasculature and is not restricted to the border of arteries and veins. In vitro assays demonstrate that both ephrinB1 and ephrinB2 have sprout-inducing activity, suggesting that the cellular response is different between neurons (repulsion, growth cone collapse) and endothelial cells (de-adhesion, migration).
In addition to angiogenesis there is accumulating evidence that indicates that ephrin/Eph signalling plays a role in other aspects of development of the blood system, although, at present, this is limited to expression data. The human EphB4 receptor is expressed by umbilical cord blood cells and erythroid progenitors and ephrinB2 has been shown to be expressed by stromal cells of the bone marrow (Inada et al., 1997; Sakano et al., 1996). Class A receptors EphA7 and EphA4 express in human fetal bone marrow pro-B cells (Aasheim et al., 1997) and EphA3 was originally isolated from a lymphoid tumour cell line (Wicks et al., 1992). A clue as to the function of ephrin/eph signalling comes from the observation that ephrinB2 can stimulate the proliferation of sorted EphB4-expressing umbilical cord blood cells (Sakano et al., 1996) but it will be interesting to see if the migration of hematopoietic precursors is also affected.

(5) A potential role for Eph receptor/ ephrin signalling in limb development

The chick EphA4 gene is expressed in a spatially regulated manner in the developing chick wing and leg buds and in the forming feather and scale primordia (Patel et al., 1996). In the limb bud, the gene expresses in the distal regions at a time when this area, the progress zone, is full of undifferentiated and dividing cells. Expression then becomes restricted more posteriorly, begins to downregulate and remains only in the forming tendons. This expression is precise and it is shown in this study that the signals known to regulate limb pattern, such as retinoic acid, FGF2 and FGF4 and BMP-2, also regulate EphA4 expression. As yet there is no indication of the role played by EphA4 in limb morphogenesis and the EphA4 mutant mouse does not show defects in limb morphogenesis (Andrew Boyd, personal communication). In the chick, EphA7 shows a highly dynamic expression pattern in the dorsal mesenchyme of developing limbs adjacent to the routes of growing axons, suggesting a role for EphA7 in dorsal limb patterning and/or axon guidance (Araujo et al., 1998).

(C) A ROLE FOR EPH SIGNALLING IN CELLULAR TRANSFORMATION?

There are a number of studies that have shown a link between Eph signalling and cell transformation, although there is as yet no firm indication of a role for Eph signalling in any cancer cell. It is, however, clear that Eph receptors are non-mitogenic when expressed in heterologous cells (Brambilla et al., 1995). There is sufficient weight of evidence for overexpression in human tumours and tumour cell lines to indicate a role for Eph/ephrin signalling in cell transformation and it is likely that these roles are linked to those controlled by Eph/ephrins during development, including de-adhesion, relief of contact inhibition and motility. These are key events in processes such as metastasis and tissue invasion by cancer cells.

The first member of the Eph family of receptor tyrosine kinases, Eph, was isolated by hybridisation to a human genomic library using a probe to the kinase domain of the viral oncogene v-fps (Hirai et al., 1987). It was shown in this first study by northern blot analysis that Eph is overexpressed in a number of human tumours including colon carcinoma, lung adenocarcinoma, mammary carcinoma and hepatocyte carcinoma. Since then, expression of a number of other receptors has been analysed in a range of cancer cell lines. EphA4 and EphB2 are expressed in a range of tumours and tumour cell lines that have neuronal, glial, epithelial and fibroblastic and epithelial characteristics and, in some of these, they are phosphorylated on tyrosine indicating activity (Soans et al., 1994, Valenzuela et al., 1995). EphB3 is also expressed in a human epidermoid carcinoma cell line (Bohme et al., 1993) The human EphA3 receptor was originally isolated using an antibody to a cell surface component of a pre-B acute lymphoblastic leukemia cell line (Sajjadi et al., 1991).

Finally, the ligand ephrinA1 and the receptor to which it can bind, EphA2, are both overexpressed in melanomas and ephrinA1 stimulated the growth of EphA2-expressing melanoma cell lines (Easty et al., 1995). These results suggest that ephrinA1 could function as an autocrine growth factor for melanoma cells. However, there is now clear evidence that Eph/ephrin signalling may be involved also in inhibiting mitogenic pathways. One of the binding partners of EphA2 receptors is Slap, a novel SH3-SH2 Src-like adaptor protein (Pandey et al., 1995). Slap appears to be a general suppressor of cell growth by antagonising Src signalling (Roche et al., 1998). Transient expression of Slap in fibroblasts by
interactions or through a link to other cell surface adhesion mechanisms. These results focus attention on the amount of ligand that is presented to a receptor-expressing cell and maybe the beginning of an explanation of how cells respond in specific ways when encountering fields of cells expressing graded distributions of ligand such as in the retinotectal system described above.

(2) The significance of the domain structure of Eph receptors is beginning to be understood

The Eph receptors have a standard structure, which is illustrated in Fig. 3. They have an uninterrupted catalytic domain intracellularly and a cysteine-rich domain and two fibronectin type III repeats in the extracellular ligand-binding region. At the extracellular N terminus, there is a globular domain, which has recently been shown to be responsible for specificity of ligand binding (Labrador et al., 1997). This was shown by creating a series of deletion and domain substitution mutants of EphB2 and examining their binding characteristics of alkaline-phosphatase-tagged proteins to ephrinB2. In domain deletion experiments, only EphB2 ectodomains containing the N-terminal globular domain bound to ephrinB2. By switching the N-terminal globular region of EphB2 with the corresponding domain in the ectodomain of the orphan receptor, EphB5, it was shown that the N-terminal globular domain was sufficient to confer ephrinB2-specific binding. Also, the globular domain of EphA3 renders the EphB2 receptor competent to bind to the class A ephrinA2. Furthermore, using transformation of NIH 3T3 cells with chimeric receptors in which the ectodomain of EphB2 was fused to the intracellular domain of the TrkB receptor tyrosine kinase as an assay, it was shown that the N-terminal globular domain is sufficient to trigger ephrinB2-dependent signalling. These results show conclusively that ligand-binding specificity resides in the N-terminal globular domain. Recently, the crystal structure of the N-terminal globular domain of EphB2 was solved (Himanen et al., 1998). The domain folds into a compact jellyroll β-sandwich composed of two antiparallel β-sheets and has structural similarities with the carbohydrate-binding domain of lectins and influenza virus hemagglutinin. Structure-based mutagenesis identified an extended loop packed against the concave β-sandwich surface as important for ligand-binding and subclass specificity.

Adjacent to the N-terminal domain is a cysteine-rich region of unknown function and two fibronectin type III repeats. Such fibronectin type III repeats appear in ectodomains of numerous cell adhesion molecules, receptor tyrosine kinases and receptor tyrosine phosphatases, and may be involved in dimerisation. In fact, incubation of cells with divalent complexes of the two fibronectin type III repeats of EphA3 caused ligand-independent EphA3 receptor autophosphorylation suggesting the presence of a dimerization motif (Lackmann et al., 1998). It was suggested that Eph receptor activation occurs by a two-step mechanism, with distinct ligand binding via the N-terminal globular domain followed by ligand-independent receptor-receptor oligomerization.

Next comes the transmembrane domain followed by the C-terminal intracellular region of the protein. This intracellular part of the protein includes the kinase domain. A highly conserved motif containing two tyrosine residues is found in the juxtamembrane intracellular region of all Eph receptors (Ellis et al., 1996; Holland et al., 1997). These tyrosine residues...
are also major in vitro autophosphorylation sites for EphA4 (Ellis et al., 1996) and EphB2 (Holland et al., 1997) and are likely to be important for intracellular signalling. It has been shown that a number of SH2 domain cytoplasmic proteins bind to the juxtamembrane region of the receptor when it is activated. These include the Src-like tyrosine kinases p59fyn and p60src, which bind to this region in EphA4 (Ellis et al., 1996) and EphB2, respectively (Zisch et al., 1998). The Ras GTPase-activating protein (RasGAP) binds through its SH2 domain to tyrosine phosphorylated EphB2, as does a 62-64 kDa protein p62dok and the SH2/Sh3 domain adaptor protein Nck. It is likely that the RasGAP, p62dok and Nck proteins form a complex bound to the juxtamembrane region of EphB2 and they potentially link signalling to control of cytoskeletal dynamics (Holland et al., 1997; Bruccner and Klein, 1998).

C-terminal to the kinase domain, a conserved region of 60-70 amino acids is present in all Eph receptors and was identified as a sterile alpha motif (SAM) domain (Schultz et al., 1997). An invariant tyrosine located within the SAM domain of EphB1 is required for binding of the Grb10 adaptor protein (Stein et al., 1998). It is of interest that Grb10 shares homology with a Caenorhabditis elegans gene product thought to be involved in neural cell migration. Grb10 has been shown not to interact with the EphA2 cytoplasmic domain (Pandey et al., 1995a) so this is not a common feature of all Eph receptors and highlights the point that different downstream responses may result from signalling through different Eph receptors. The tyrosine within the SAM domain of EphB1 is also required for binding of LMW-PTP whose recruitment correlates with functional responses, such as endothelial capillary-like assembly and cell attachment after stimulation with higher order ephrin clusters (Stein et al., 1998). Recently, the X-ray crystal structure of the SAM domain in the EphA4 receptor was solved. The structure reveals a homodimer of two ‘lobster claw’-shaped subunits. In contrast to many other protein domain interactions, the interaction surface consists of the termini, which interdigitate in a pincer-like manner with the termini of the other subunit (Stapleton et al., 1999). It is speculated that the SAM domain influences, either positively or negatively, the formation of Eph receptor dimers in addition to their suggested function in recruiting signalling partners.

Finally, a PDZ-binding motif that interacts with PDZ domain proteins is present at the C-terminal tail of Eph receptors (PDZ for postsynaptic density protein, discs large, zona occludens ; Sheng, 1996). In line with their known interactions with synaptic membrane proteins, PDZ domain proteins were found to cluster and co-localize with Eph receptors at synapses of cultured hippocampal neurons (Torres et al., 1998). Some PDZ domain proteins become tyrosine phosphorylated when complexed with Eph receptors (Torres et al., 1998) and an intact Eph kinase domain appears to be required for the interaction (Hock et al., 1998). Interestingly, a functional PDZ-binding motif is also present at the C terminus of transmembrane ephrinB proteins and at least one multi-PDZ domain protein, Glutamate-Receptor-Interacting-Protein (GRIP) was shown to interact with both an EphB receptor and ephrinB ligands (Torres et al., 1998; Bruccner et al., 1999). Moreover, ephrinB ligands are found in lipid-enriched raft microdomains, which are thought to function as platforms for the localized concentration and activation of signaling molecules. GRIP proteins are moved into these rafts by binding to ephrinB1. Although these findings are yet to be confirmed in primary neurons, they suggest that GRIP adaptor proteins function to provide a scaffold for the assembly of a multi-protein signaling complex downstream of ephrinB ligands (Bruckner et al., 1999).

(3) Variation in protein structure may underlie differences in function

The structure of the receptor protein varies in some cases and forms are generated that may function negatively in a signalling context. For example, the chicken EphB2 message exists in three forms, the full-length protein, which has the same basic structure as other Eph receptors, a form in which an insertion of 48 nucleotides is made in the juxtamembrane region and a form encoding a soluble protein consisting only of the extracellular region (Connor and Pasquale, 1995; Sajjadi and Pasquale, 1993). Such a truncated form lacking the kinase domain, also exists for rat EphA7 (Valenzuela et al., 1995) and for mouse EphA3 (Sajjadi et al., 1991). EphA5 and EphB3 also have insertions in the juxtamembrane region (Maisonpierre et al., 1993). The function of these variants is not known, although in situ hybridisation and northern blot analysis has demonstrated that they are expressed. Furthermore, they are all accountable in terms of the known exon/intron structure of EphB2, which suggests that they are all formed by differential splicing events (Connor and Pasquale, 1995). It is tempting to suggest that the kinase inactive forms could function as dominant negative proteins because engineered forms of receptors work in this way when overexpressed (see for example Xu et al., 1995, 1996). The insertions in the juxtamembrane region could affect downstream signalling because this region contains conserved tyrosine residues involved in binding to SH2 domain adaptor proteins (Ellis et al., 1996; Holland et al., 1997).

The identification and analysis of the genes for Eph receptors and ephrins and chromosomal mapping will also reveal interesting information concerning their regulation and evolution. This is evident from initial studies where the high level of conservation of exon/intron structure have been demonstrated for ephrinA class ligands and EphB class receptors (Cerretti et al., 1996; Cerretti and Nelson, 1998; Connor and Pasquale, 1995).

(E) CONCLUSIONS

Eph receptors and ephrins are dynamically expressed during development of a range of vertebrate species and have been isolated in C. elegans (George et al., 1998). Examination of their function with regard to processes as diverse as segmentation of the somites and rhombomeres, the formation of blood vessels, axonal guidance, migration of the neural crest and metastasis of transformed cells indicates that signalling through these receptor tyrosine kinases and possibly also through the class B ephrins controls cellular morphology. In the case of somite and rhombomere segmentation boundary formation is achieved by the reciprocal spatial expression of receptor and ligand. With regard to the control of cell movement, in processes such as guidance of growth cones and migration of neural crest cells the function of Eph receptor/ephrin signalling may not be based on reciprocal
expression but on subtle changes in level of expression and possibly on the degree of receptor clustering. It is not yet clear how a growth cone belonging to a chick retinal ganglion cell, for example, transduces a signal based on interacting with a graded level of ephrin presented by the tectal cells. Similarly, during the differentiation of arteries and veins, they all express ephrins and Eph receptors but at different levels.

The questions concerning the intracellular pathways linking Eph receptor/ephrin signalling to the cytoskeleton, the principle mediator of changes in cell form, remain. Our understanding of the specificity of cellular responses to this large group of receptors will be resolved as the intracellular pathways are defined and the structural features of the receptor and ligand proteins are understood. Little is known about the pathways are defined and the structural features of the receptor and ligand proteins are understood. Little is known about the dynamic nature of their expression and the changes in upstream regulation of the Eph receptor and ephrin genes – the large group of receptors will be resolved as the intracellular pathways are defined.

Whatever the answers to these questions are it is already clear that Eph/ephrin signalling lies at the heart of morphogenesis.

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REFERENCES


