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Eph Receptors and Ligands Comprise Two Major Specificity Subclasses and Are Reciprocally Compartmentalized during Embryogenesis

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Summary

We report that the many Eph-related receptor tyrosine kinases, and their numerous membrane-bound ligands, can each be grouped into only two major specificity subclasses. Receptors in a given subclass bind most members of a corresponding ligand subclass. The physiological relevance of these groupings is suggested by viewing the collective distributions of all members of a subclass. These composite distributions, in contrast with less informative patterns seen with individual members of the family, reveal that the developing embryo is subdivided into domains defined by reciprocal and apparently mutually exclusive expression of a receptor subclass and its corresponding ligands. Receptors seem to encounter their ligands only at the interface between these domains. This reciprocal compartmentalization implicates the Eph family in the formation of spatial boundaries that may help to organize the developing body plan.

Introduction

Factors that bind and activate receptor tyrosine kinases (RTKs) play key roles during development as well as in the adult (Schlessinger and Ullrich, 1992). The known RTKs can be grouped into families based on structural considerations. The Eph family of RTKs, named for its first described member (Hirai et al., 1987), is the largest known family of RTKs with at least 13 distinct members (Tuzi and Gullick, 1994). Members of the Eph receptor family display dynamic and spatially restricted expression patterns during embryogenesis, which originally suggested that they might be involved in a variety of developmental processes. For example, segmental expression of various Eph receptors such as murine Sek1 (also known as Hek8 in humans and Cek8 in chick) and Eck (also known as Sek2, Mpk5, and Myk2) in early somites and in the rhombomeres of the developing hindbrain suggested that they may be involved in formation of body segments or in regulating segment-specific characteristics (Nieto et al., 1992; Gilardi-Hebenstreit et al., 1992; Ruiz and Robertson, 1994). Expression of Eph family members in the limb and particular neural structures suggested additional roles (Nieto et al., 1992; Ganju et al., 1994; Cheng and Flanagan, 1994), with the expression of the murine Nuk receptor (also known as Hek5 and Erk in humans, Sek3 in mice, and Cek5 in chick) on initial axonal outgrowths, suggesting a role for this RTK in early axonal pathfinding or in the fasciculation stages of axonogenesis (Henkemeyer et al., 1994). Several of the Eph receptors have also generated interest because of their restricted expression to the nervous system in the adult, such as for Ehk1 (alternately dubbed Bsk or Rek7 in rats, and also known as Hek7 in humans), Ehk2, Ehk3 (with the mouse ortholog also referred to as MDK1 or Ebk and the human version Hek11), and Elk (also known as Cek6 in chick), (Lhotak et al., 1991; Maisonpierre et al., 1993; Zhou et al., 1994; Taylor et al., 1994; Valenzuela et al., 1995; Ciossek et al., 1995; Ellis et al., 1995).

Members of the Eph receptor family were all initially identified as "orphan receptors," because at the time of their identification they had no known ligands. Recently, however, protein factors that bind these receptors have been molecularly cloned at an impressive rate. B61 was initially cloned as a TNF-inducible sequence of unknown function (Holzman et al., 1990), but was subsequently recloned by a number of groups as the ligand for the Eck receptor (Bartley et al., 1994), as a binding protein for the Hek receptor (also referred to as Hek4 in humans, Mek4 in mouse, and Cek in chicken) and termed LERK1 (Beckmann et al., 1994), or as a ligand for both Eck and Ehk1 and termed Efl-1 (Davis et al., 1994). Six additional ligands for Eph receptor have been published, with each of these ligands (like B61) having been independently cloned by several different groups based on binding to different members of the Eph receptor family (Beckmann et al., 1994; Davis et al., 1994; Cheng and Flanagan, 1994; Shao et al., 1994, 1995; Kozlosky et al., 1995; Bennett et al., 1995; Winslow et al., 1995; Drescher et al., 1995; Cerretti et al., 1995; Lackmann et al., 1996); perhaps surprisingly, no ligands have been reported to bind to the original and prototypical member of this RTK subfamily, Eph. The seven published ligands comprise a family, with members sharing between 23% and 56% amino acid identity. The most striking unifying feature of

all the Eph family ligands is that they are all membrane-attached, either because they are transmembrane proteins (as for Htk-L/ELF-2/LERK5 and Elk-L/LERK2/Efl-3/Cek-5L, hereafter referred to as Htk-L and Elk-L/LERK2) or because they are bound to the surface via a glycosylphosphatidylinositol (GPI) linkage (as is the case for B61/LERK1/Efl-1, Ehk1-L/Efl-2/LERK3, LERK4, ELF-1/Cek7-L and AL-1/RAGS, hereafter referred to as B61, Ehk1-L, LERK4, ELF-1, and AL-1). While ligands for several other families of RTKs can have membrane-bound and soluble forms that are both active, the Eph family ligands are unusual in that only their membrane-bound forms are active while soluble forms are not only inactive but in fact may act as antagonists (Davis et al., 1994; Winslow et al., 1995). However, the soluble forms can be artificially activated by deliberate dimerization or higher order oligomerization, leading to the proposal that these ligands are active in their membrane-attached forms because membrane attachment normally serves to facilitate their oligomerization (Davis et al., 1994). The strict requirement for membrane attachment seems to provide for a specialized mechanism that ensures that receptor activation is coupled to direct cell-to-cell contact (Davis et al., 1994) and is consistent with findings that Eph family receptors can be highly localized to patches of cell-to-cell contact (Henkemeyer et al., 1994).

In contrast with other ectopically expressed neural RTKs such as the Trk receptors used by the neurotrophins, when Eph family receptors are ectopically expressed in non-neuronal cells they can not elicit conventional growth responses (Lhotak and Pawson, 1993; Davis et al., 1994; Brambilla et al., 1995). Because of these findings, together with the unusual requirement that the ligands act as obligate membrane-attached factors (Davis et al., 1994; Winslow et al., 1995), it may not be too surprising that emerging functional evidence (Winslow et al., 1995; Drescher et al., 1995; Cheng et al., 1995) indicates that Eph family ligands elicit responses from neurons and their precursors that are quite different from those seen in response to classical neurotrophic and survival factors such as the neurotrophins. The Eph family may instead be involved in axonal bundling or guidance, perhaps by providing repulsive signals, and has been specifically implicated as providing positional cues for establishing retinotectal projection patterns (reviewed by Tessier-Lavigne, 1995).

The very large size of the Eph family of receptors and ligands initially seemed well-suited for providing the diversity necessary to mediate distinct and specific recognition events in the nervous system and elsewhere (reviewed by Tessier-Lavigne, 1995). Early hints, however, suggested that the number of distinct binding specificities encoded by this family might be much smaller than that predicted based on family size. For example, as noted above, several of the ligands were independently identified using different Eph family receptors, indicating that the ligands could bind multiple receptors. Direct comparison has indeed shown that this can be the case, finding that particular ligands can indeed have rather similar affinities for several receptors (e.g., Davis et al., 1994; Beckmann et al., 1994; Kozlosky et al., 1995; Cheng and Flanagan, 1994; Brambilla et al., 1995). Here, we report that all the Eph family ligands identified to date, as well as most of the known Eph family receptors, can each be functionally divided into only two major specificity subclasses. Thus, one subclass of ligands binds and activates one subclass of receptors, while the second subclass of ligands binds and activates the other subclass of receptors. The composite distributions of these subclasses during embryogenesis suggest that the Eph family is involved in formation of spatial boundaries that may help to organize the developing body plan.

Results

Eph Family Ligands and Receptors Each Segregate into Only Two Major Subclasses Based on Binding Specificities

To determine the binding specificities of the various Eph family receptor and ligands, we first transiently expressed each of the ligands on the surface of COS7 cells, and then assayed for the binding of saturating concentrations of soluble receptor–antibody fusion proteins (dubbed receptor–bodies, consisting of the extracellular domain of the receptor fused to the Fc portion of human IgG1) to these cells (Figure 1A; summarized in Figure 6A). These binding studies revealed that all the Eph family ligands identified to date, as well as most of the known Eph family receptors, can each be functionally divided into only two major specificity subclasses. One subclass of ligands (Elk-L/LERK2 and Htk-L, as well as a novel ligand dubbed Elk-L3 to be described in detail elsewhere; Gale et al., 1996) binds to one subclass of receptors (Elk and Nuk), while the other subclass of ligands (B61, Ehk1-L, LERK4, AL-1, and ELF-1) binds to the other subclass of receptors (Eck, Ehk1, Ehk2, Ehk3/MDK1, and Sek1); the Eph receptor proved unique in that it only binds to a single ligand, B61. Interestingly, the ligand subclasses as defined by binding specificities also correspond to subclasses defined by the type of membrane linkages used by the ligands, that is, all members of one subclass of ligands (Elk-L/LERK2, Htk-L, and Elk-L3) are transmembrane proteins, while all the members of the other subclass (B61, Ehk1-L, LERK4, AL-1, and ELF-1) are GPI anchored. Furthermore, the binding specificity subclasses of both the ligands and receptors also correlate with groupings that can be made based on homologies displayed by these proteins (see Figures 6A and 6B).

Although we did not directly test the interaction of Hek/Mek4 with this panel of ligands, Hek/Mek4 is most homologous to Ehk2, and others have shown that Hek/Mek4 can interact with B61 (Beckmann et al., 1994), Ehk1-L/LERK3 and LERK4 (Kozlosky et al., 1995), and ELF-1 (Cheng and Flanagan, 1994; Lackmann et al., 1996). Thus, Hek/Mek4 can be included among the receptor subclass that includes Eck, Ehk1, Ehk2, Ehk3/MDK1, and Sek1. Similarly, it has previously been shown that the Htk (also known as Myk1 and MDK2) receptor binds to Htk-L (Bennett et al., 1995; Bergemann et al., 1995) and the chicken ortholog (Cek10, and also known as Sek4 and MDK5 in mouse) of Hek2 binds Elk-L/LERK2 (Brambilla et al., 1995) and Htk-L (Bergemann et al., 1995), indicating that Htk and Hek2 should be grouped with the Elk and Nuk subclass of receptors (Figure 6A; also see below for further data in this regard).

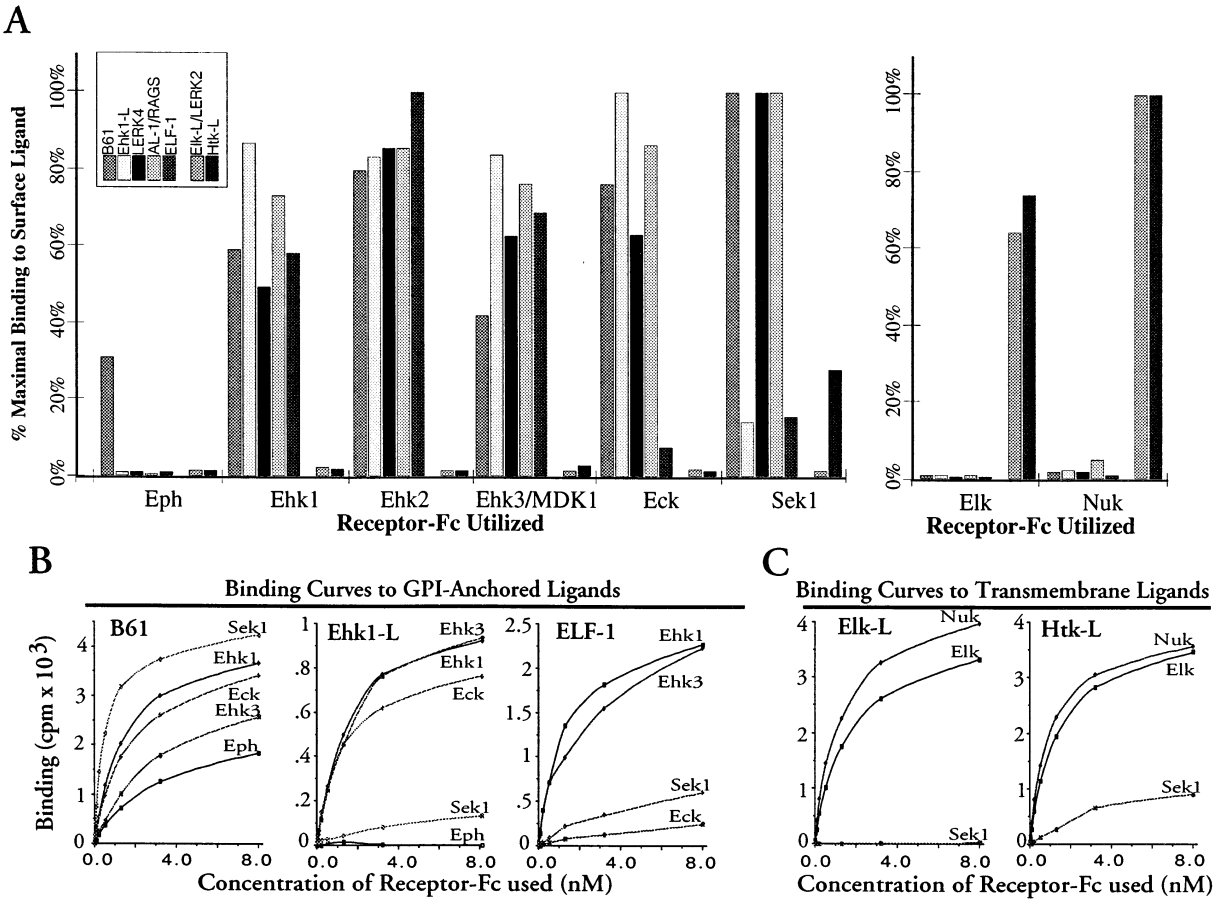

Figure 1. Binding Analyses Reveal Two Major Specificity Subclasses for the Eph Family

(A) Saturation binding of receptor–Fc to surface ligands. Binding of the indicated receptor–bodies, each at saturating concentrations, to a panel of transiently transfected COS cells expressing the indicated cell-surface bound ligands.

(B and C) Indirect scatchard analysis of receptor/ligand binding. Analysis performed by measuring binding of varying concentrations of receptor–Fc fusions, as labeled on each binding curve, to transiently expressed surface bound ligand, indicated in the upper left corner of each graph.

Differences in Binding Specificities and Affinities of Members of the Same Subclass

Although different members of a particular subclass appear to have remarkably similar overall binding specificities as determined using saturating concentrations of receptor–bodies (Figure 1A), notable differences can also be seen. For example, Eck and Sek1 bind rather poorly to ELF-1, and Sek1 binds poorly to Ehk1-L, although Eck and Sek1 bind well to all the other GPI-anchored ligands. Furthermore, Sek1 appears to "cross" subclasses by exhibiting appreciable binding to the transmembrane ligand Htk-L. To explore further such differences in the binding specificities and affinities of members of the same subclass, sets of ligand–receptor pairings were selected for indirect scatchard analysis performed by measuring binding of varying concentrations of receptor–bodies (Figures 1B and 1C). Such binding analysis did indeed reveal clear differences in binding specificities and affinities of members of the same ligand or receptor subclass (Figure 2). Thus, B61 was bound with decreasing affinity by Sek1, Ehk1, Eck, Ehk3, and Eph; there was an approximately 10-fold difference in the binding affinity of B61 in the case of its highest affinity interaction (to Sek1, K_d ~0.395 nM) compared with its lowest affinity interaction (to Eph, K_d ~2.67). There were similar variations in the binding affinities of Ehk1-L and ELF-1 by the various receptors, except that each displayed different relative preferences for the various receptors.

These data demonstrate that although members of a particular ligand subclass exhibit similar overall patterns of binding that are generally restricted to members of the corresponding subclass of receptors, each ligand displays a different set of preferences for these receptors, with the affinity of interaction ranging from the subnanomolar range to undetectable. Sek1 and Htk-L provide examples that "cross" subclasses by binding to each other with appreciable affinity, while Eph provides an example of a receptor with a very limited binding repertoire restricted to B61.

Eph Family Ligands Induce "Subclass-Specific" Receptor Activation

To determine whether the subclasses defined based on binding specificities correspond to functionally relevant groupings, we examined whether Eph family ligands

Receptors And Transmembrane Ligands

Rec\Lig Pair	Elk-L	Htk-L
Elk	1.16 nM	1.12 nM
Nuk	0.81	0.77
Sek1	n/a	8.60

Receptors And GPI-Anchored Ligands

Rec\Lig Pair	B61	Ehk1-L	ELF-1
Ehk1	1.19 nM	1.78 nM	1.31 nM
Ehk3/MDK1	2.41	1.47	1.03
Eck	1.33	0.95	20.10
Sek1	0.395	3.0	3.96
Eph	2.67	n/a	n/d

Figure 2. Calculated K_ds for Receptor–Fc Binding to Surface Ligands

Results of indirect scatchard analysis for the receptor/ligand pairs in Figures 1B and 1C are shown in matrix form and are expressed as K_ds (nM); n/a, too low to be determined accurately; n/d, not determined.

could indeed generally activate members of the corresponding subclass of receptors to which they could bind. Thus, deliberately clustered soluble ligands were assessed for their ability to induce phosphorylation of several members of the Eph receptor family; for these experiments, we employed MG mouse fibroblast cells stably expressing the Elk receptor (MG-Elk cells), COS1 monkey kidney cells endogenously expressing Nuk receptors, NIH 3T3 mouse fibroblast cells endogenously expressing Hek2 receptors, or CHP100 human neuroepithelial cells endogenously expressing Ehk1 receptors. The receptor phosphorylation results proved to be entirely consistent with the above binding studies. Thus, the transmembrane class of ligands (Elk-L/LERK2 and Htk-L) induced phosphorylation of Elk, Nuk and Hek2, while the GPI-anchored class of ligands (B61, Ehk1-L, Efl-4, or AL-1) did not (Figure 3, top). In contrast, the GPI-anchored ligands all induced activation of Ehk1 (and also Ehk2 and Eck; data not shown) while the transmembrane ligands did not (Figure 3, bottom). The findings for Hek2 also confirm the assumption made above, based on the binding specificities of the presumed chicken ortholog of Hek2 (Brambilla et al., 1995), that the mammalian Hek2 receptor can be placed into the group of receptors interacting with transmembrane ligands that also includes Elk, Nuk, and Htk.

Subclass Groupings Are Consistent with Binding Profiles in Whole Embryos

We next attempted to determine whether the Eph family ligand and receptor subclasses, defined based on the in vitro binding and receptor activation assays described above, were relevant to the specificities of these ligands and receptors for their in vivo counterparts. Toward this end, we stained whole embryos (embryonic day ~10.5) with either soluble receptor–bodies to define the embryonic distribution of their ligands, or soluble "ligand–bodies" (soluble ligand–antibody fusion proteins consisting of the soluble portion of the ligand fused to the antibody Fc domain) to define the embryonic distribution of their corresponding receptors. Consistent with the

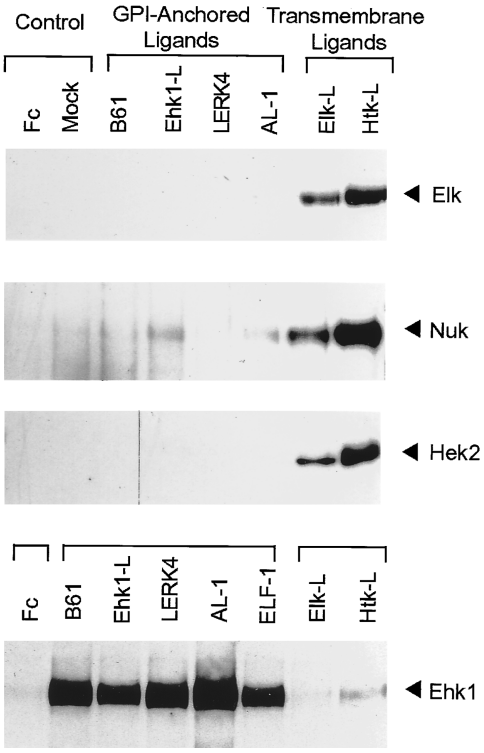

Figure 3. Eph Family Ligands Induce "Subclass-Specific" Receptor Activation

Receptor activation assayed by anti-phosphotyrosine immunoblotting of receptor precipitates from MG cells transfected with an expression construct to allow for stable expression of the Elk receptors, COS cells endogenously expressing Nuk receptors, NIH 3T3 cells endogenously expressing Hek2 receptors, or CHP100 cells endogenously expressing Ehk1 receptors. Cells stimulated with either control reagents (Fc alone, or Mock treated) or clustered ligand–bodies, as indicated.

subclass groupings determined above by the in vitro binding and phosphorylation studies, receptors in a particular subclass all identified similar ligand patterns in the embryo, while ligands in a particular subclass also all detected rather uniform receptor profiles (Figure 4). However, consistent with the differences in relative affinities of members of a particular subclass for their counterparts, there were also occasional notable differences in the patterns identified by members of the same receptor or ligand subclass. Strikingly, the distribution of a given receptor subclass often seemed to be complementary to the distribution of its corresponding ligand subclass, suggesting that these receptors and their ligands mark or define boundaries in the embryo (this point addressed in detail below).

Elk and Nuk receptor–bodies, representing receptors interacting with transmembrane ligands when assayed on cell lines, both detected similar ligand patterns in the embryo (Figure 4B) that consisted of staining in the forebrain, lateral nasal process, dorsal midbrain/tectum, anterior dorsal hindbrain, eye regions, a dorsal stripe along the entire spinal cord, distinctive portions of the branchial arches, proximal limb bud, tail somites, and umbilical cord. Ligand–bodies representing the corresponding subclass of ligands, including Elk-L/LERK2

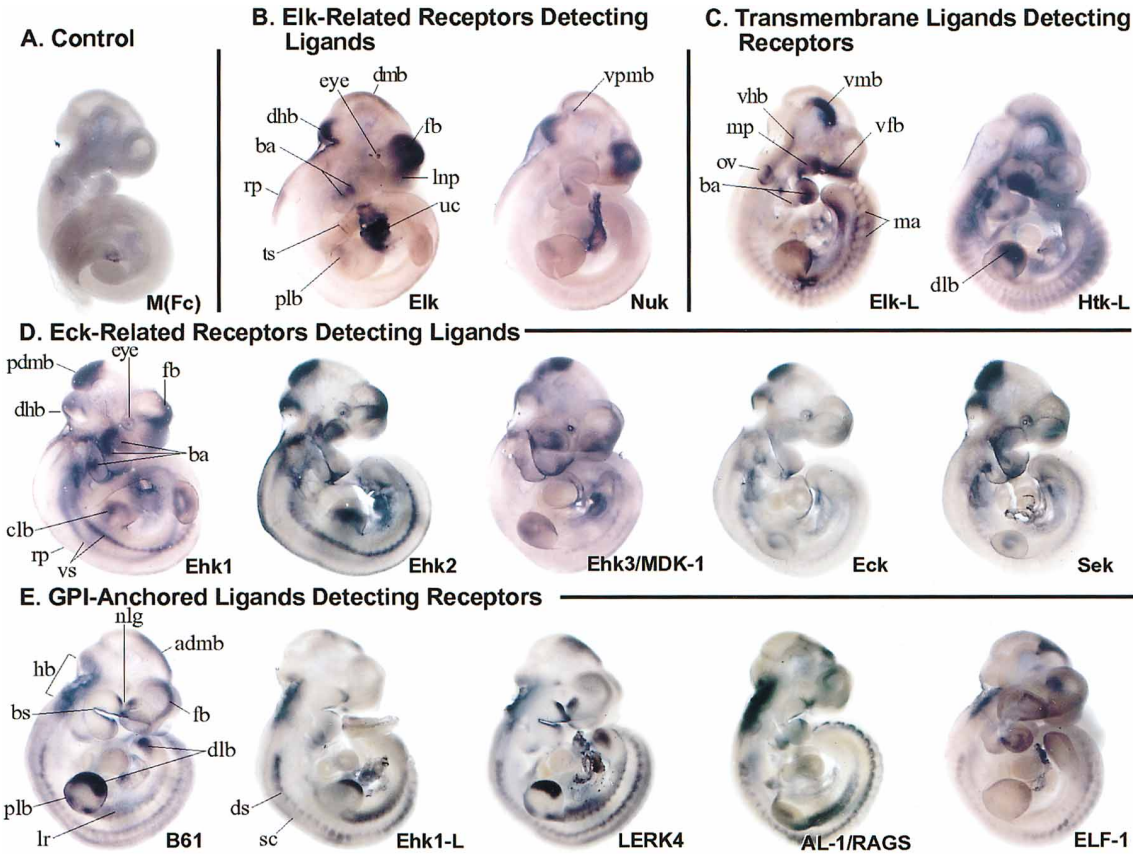

Figure 4. In Situ Binding Specificities of Eph Family Receptors and Ligands

Receptor–Fc and ligand–Fc fusions were bound to whole 10.5 dpc embryos, and sites of binding were visualized by immunohistochemistry using an alkaline phosphatase–conjugated anti-human Fc secondary antibody.

(A) Embryos bound with Fc alone, showing no detectable background binding.

(B) Embryos bound with Elk–Fc or Nuk–Fc to localize the in situ distribution of their ligands.

(C) Embryos bound with Elk-L/LERK2–Fc and Htk-L–Fc, two transmembrane ligands, to localize the distribution of their receptors.

(D) Embryos bound with Fc fusions to the Eck-related receptors Ehk1, Ehk2, Ehk3/MDK-1, Eck, and Sek1 to localize the in situ distribution of their ligands.

(E) Embryos bound with Fc fusions of the GPI-anchored ligands (B61, Ehk1-L, LERK4, AL-1/RAGS, and ELF-1) to localize distribution of their receptors.

Abbreviations: forebrain (fb), ventral forebrain (vfb), dorsal midbrain (dmb), anterior dorsal midbrain/pretectum (admb), posterior dorsal midbrain/tectum (pdmb), ventral midbrain (vmb), hindbrain (hb), dorsal hindbrain or cerebellar anlage (dhb), ventral hindbrain (vhb), spinal cord (sc), roof plate of spinal cord (rp), motor axons (ma), otic vesicle (ov), lateral nasal process (lnp) regions of the branchial arches (ba), nasolacrimal groove (nlg), slits/grooves of branchial arches (bs), maxillary process of the first branchial arch (mp), umbilical cord (uc), a body proximal band in the limb buds (plb), distal limb bud (dlb), central limb bud region (clb), dorsal region of the somites (ds), ventral region of the somites (vs), the lateral mesodermal ridge (lr), and a region of the newly formed tail somites (ts).

and Htk-L, both identified a characteristic receptor pattern distinguished by staining in the ventral region of the midbrain and diencephalon, the most ventral region of the hindbrain, otic vesicles, the maxillary process and branchial arches, and in segmentally expressed dorsoventral stripes, which correspond to motor neuronal processes (Figure 4C; data not shown). A panel of receptor–bodies corresponding to the other subclass of receptors, that bind GPI-linked ligands in vitro, all identified largely similar ligand patterns in the embryo that were quite distinct from the ligand patterns detected by receptor–bodies representing the other subclass (Figure 4D). The ligand patterns detected by this panel of receptor–bodies was highlighted by staining in regions including the eye, the surface of the branchial arches (i.e., not in the slit/groove area of the arches), forebrain, dorsal posterior midbrain, dorsal anterior hindbrain, roof plate of the spinal cord, in a ventral stripe along the somites, and within the limb bud in regions apparently exclusive of both the tip and the region most proximal to the body (Figure 4D and see below for further details). Finally, ligand–bodies representing the GPI-anchored ligands all exhibited remarkably similar binding patterns representing sites of expression of their receptors, which often seemed reciprocal to the distributions of the ligands themselves (Figure 4E and see below for further details). Receptors were detected in the forebrain, dorsal anterior midbrain/pretectum, in the hindbrain in the area of the rhombomeres, in the branchial arches within the slit/groove area exclusive of the surface of the arches and in the nasolacrimal groove, in a stripe along the dorsal region of the somites, and along the lateral mesodermal ridge (Figure 4E and see below). In the limb, sites of receptor expression were revealed in the distal

limb bud as well as in a body proximal location within the bud (Figure 4E and see below). Receptor expression was also prominently detected in the tail bud (not visible in the figure).

Despite the similar overall patterns detected by members of the same subclass, members did exhibit occasional notable differences, consistent with the above described binding assays, demonstrating that members of a given subclass could indeed display distinctive differences in their relative affinities for their counterparts. For example, in contrast with B61 ligand–bodies, ELF-1 ligand–bodies did not appreciably stain the tips of limb buds or tail buds and appeared to detect a more posterior region of the hindbrain (Figure 4E); the particular inability of ELF-1 ligand–bodies to stain limb buds can be explained by previous findings (Ganju et al., 1994; Cheng and Flanagan, 1994) that Eck and Sek1, which we have shown bind well to all GPI-linked ligands except for ELF-1, are localized to the limb bud tip. Eph receptor–bodies did not show detectable binding to whole embryos (data not shown).

Reciprocal Expression of Eph Receptors and Ligands Compartmentalize the Developing Body Plan and Suggest Dynamic Roles in Boundary Formation

The embryonic distributions of Eph receptors and ligands, as revealed by receptor body and ligand body staining experiments described in the previous section, suggested that these molecules might be involved in subdividing the body plan and perhaps in defining embryonic boundaries. Initial impressions that Eph receptors and ligands might be forming boundaries came from comparing distributions in the developing brain. Thus, receptors noted in the ventral midbrain by Elk-L/LERK2 and Htk-L ligand–bodies appeared to be clearly bounded on either side by ligands expressed in the forebrain and the ventral posterior midbrain/hindbrain border as detected by Elk and Nuk receptor–bodies (compare Figures 4B and 4C); consistent with reciprocity in their expression, the ventral distributions of this class of receptors in the forebrain, midbrain and hindbrain are contrasted by dorsal locations of the corresponding ligands in these brain regions. Similarly, GPI-linked ligands and their receptors also seemed to define a complementary boundary between the dorsal posterior midbrain and the dorsal anterior midbrain (compare Figures 4D and 4E).

More apparent and dramatic examples of complementary domains and boundaries involving this subclass of ligands and their receptors were noted in the limb buds, in the branchial arches, in the spinal cord, and in somite regions. These domains and boundaries appeared quite dynamic, with dramatic changes occurring as development proceeds. For example, in the limb buds at embryonic day 10.5, GPI-anchored ligands were only detected in the central portion of the limb (prospective zeugopod) and were bounded by receptors expressed at the distal tip (prospective autopod) as well as in body-proximal areas (prospective stylopod; compare high power views provided in Figure 5A). Strikingly, reciprocal expression of receptors and ligands was also noted later in limb development, forming different but still dramatic boundaries. Thus, in the distal limb at embryonic day 13.5, the GPI-anchored class of ligands could only be detected between the developing digits and not in the apical ectodermal ridge (AER), while receptors for these ligands were only noted in the digits themselves and in the AER (Figure 5B).

In the branchial arches, receptors for GPI-linked ligands were detected within the slit regions of the arches, while the ligands were noted primarily on the surface but not within the slit area of the arch (Figure 5C). Complementary binding patterns were also observed in the somites where the GPI-linked ligands appeared to be expressed in the ventral region of the somites, bounded on either side by receptors expressed in the dorsal region of the somite, as well as in the lateral ridge mesoderm (Figures 4D and 4E). To explore further the impression that the GPI-linked ligands and their receptors formed complementary boundaries in the somite regions of the embryos, the stained embryos were sectioned to allow for a more detailed comparison of ligand and receptor distributions in the trunk. Examination of such sections confirmed the precisely reciprocal nature of the expression patterns of these ligands and their receptors (Figure 5D). Thus, receptors for GPI-linked ligands were found in the dorsomedial region of the somite and were directly bounded by ligands expressed in dorsal root ganglion, the ventrolateral region of the somite as well as the lateral ridge mesoderm. Within the spinal cord, multiple striking boundaries could also be noted. Thus, receptors could be seen across the ventral spinal cord, exclusive of the floor plate. This receptor expression appeared to be capped by a thin layer of ligand expression, which was in turn overlaid by dense receptor expression that extended up to, but did not include, the roof plate area, which in turn strongly expressed the ligands.

Altogether, this analysis reveals that members of a particular Eph receptor subclass and their corresponding ligands are expressed in reciprocal and apparently mutually exclusive patterns that subdivide the embryo into clear domains that seem to form precise and dynamic boundaries in many different embryonic structures. Interestingly, it appears as if receptors may only encounter their ligands at the boundaries between the domains, although it remains possible that receptors and ligands interact within broader regions that appear to form sharp boundaries only because coexpressed partners interact and thus “mask” detection of each other. It is worth noting that distribution analysis of individual members of a subclass would not have readily revealed these domains, since each member accounts for only a portion of the composite pattern representing an entire subclass (data not shown; A. F. et al., unpublished data).

Discussion

Our results demonstrate that all the Eph family ligands identified to date, as well as most of the known Eph family receptors, can each be functionally divided into only two major specificity subclasses (Figure 6A). The

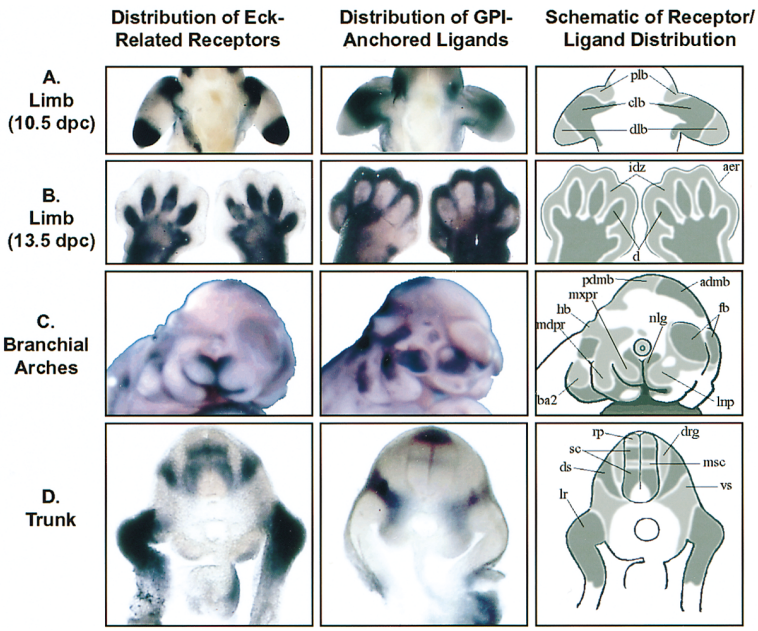

Figure 5. GPI-Anchored Ligands and Their Receptors Compartmentalize the Developing Body Plan and Form Dynamic Boundaries during Embryogenesis

Embryos were stained with an Fc fusion of a representative GPI-anchored ligand (B61, LERK4, or AL-1/RAGS) to reveal the composite distribution of the receptors binding this ligand subclass (left panels), or with an Fc fusion of one of these receptors (Ehk1 or Ehk2) to reveal the composite distribution of the GPI-anchored ligands themselves (middle panels). Binding was performed as in Figure 4, but embryos are shown at high power and/or sectioned through the trunk to allow for optimal comparison of receptor and ligand distribution. Schematic representations of the reciprocal patterns observed are provided in right panels.

(A) Forelimb buds of 10.5 dpc embryos depicting receptor expression in proximal and distal limb bud regions (plb and dlb, presumptive autopod and stylopod) with reciprocal ligand expression in the central limb bud region (clb, presumptive zeugopod).

(B) Developing hands of 13 dpc embryos reveals receptor expression in the digits (d) and in the apical ectodermal ridge (aer) and reciprocal ligand expression in the interdigital zone (idz).

(C) Views of the branchial arch region of embryos reveal receptor expression within the grooves of the branchial arches and in the nasolacrimal groove and olfactory pits, while ligand expression is reciprocally noted on the surface of the branchial arches exclusive of the grooves and in the lateral nasal process exclusive of the nasolacrimal groove and olfactory pits.

(D) Embryos sections from immediately posterior to the forelimb, and the trunk region viewed in cross section reveals complementary localization of receptors and ligands within the spinal cord, somites, and lateral mesoderm. Note that ligand expression within the dorsal root ganglion (drg) adjacent to the spinal cord, as noted in the schematic summary, was only noted in sections containing the drg (weak staining noted), although the drg is not visible in the section shown in the left panel. Abbreviations are as above and for Figure 4, with the following additions: maxillary process of the first branchial arch (mxpr), mandibulary process of the first branchial arch (mdpr), second branchial arch (ba2), mid stripe in spinal cord (msc).

ligand subclasses correlate with the manner in which the ligands are anchored to the membrane. Thus, Elk-L/LERK2, Htk-L, and Elk-L3 are members of the "transmembrane class" of ligands, while B61, Ehk1-L, LERK4, ELF-1, and AL-1 are members of the "GPI-anchored class" of ligands. Not surprisingly, these ligand subclasses also reflect the degree of homology the ligands share with each other, with members of a subclass being most related (Figure 6B). Similarly, the two receptor subclasses defined based on their binding and activation specificities also generally reflect the relatedness of their extracellular domains, with the Elk-related subclass (comprised by Elk, Nuk, Hek2, and Htk) being rather specific for "transmembrane class" ligands and the Eck-related subclass (consisting of Eck, Ehk1, Ehk2, Ehk3/MDK-1, Sek1, and Hek) for "GPI-anchored" ligands (Figure 6). The only known Eph-related receptors that have not been grouped according to ligand specificity now include the Cek9 (which appears most homologous to the Elk subclass) and Eek receptors; full ectodomain sequences have not yet been reported for either of these receptors. The division of both Eph family receptors and ligands into the two major subclasses defined here seems quite important biologically, since the observed binding specificities have been preserved evolutionarily in all cases where the same receptor has been studied from both mammals and lower vertebrates.

Eph fits outside of the major subclasses because it has a very limited binding repertoire, thus far restricted only to B61; it remains possible that this receptor may bind to yet undescribed ligands and may thus comprise an entirely separate subclass. Sek1 and Htk-L "cross" subclasses by binding to each other with appreciable affinity ($K_d \sim 8.6$ nM). An additional recently cloned ligand of the transmembrane subclass, Elk-L3, also binds Sek1 as well as Elk and Nuk, extending the notion that Sek1 "crosses" subclasses (Gale et al., 1996; Figures 6A and 6B). Within a particular ligand subclass, individual members clearly display a different set of preferences for their corresponding receptors, with the affinity of interaction ranging from the subnanomolar range to undetectable. In contrast with soluble ligands, whose affinity for their receptors reflects whether this binding can occur at physiologic levels of the ligand, it is much more difficult to predict whether particular Eph family receptor–ligand pairings are biologically relevant based on their affinity of interaction. This is because Eph receptor–ligand interactions occur at cell-to-cell interfaces, and therefore cooperative interactions due to multiple simultaneous receptor–ligand pairings may stabilize even weakly interacting partners. Thus, while binding and receptor activation studies reveal whether an exogenously provided ligand can pharmacologically interact with a particular Eph receptor, conclusions that this ligand and receptor normally pair in vivo will probably require that they are shown to colocalize to adjacent cells in vivo.

Why is it that such a large family of ligands has such a limited set of binding specificities? Unlike soluble li-

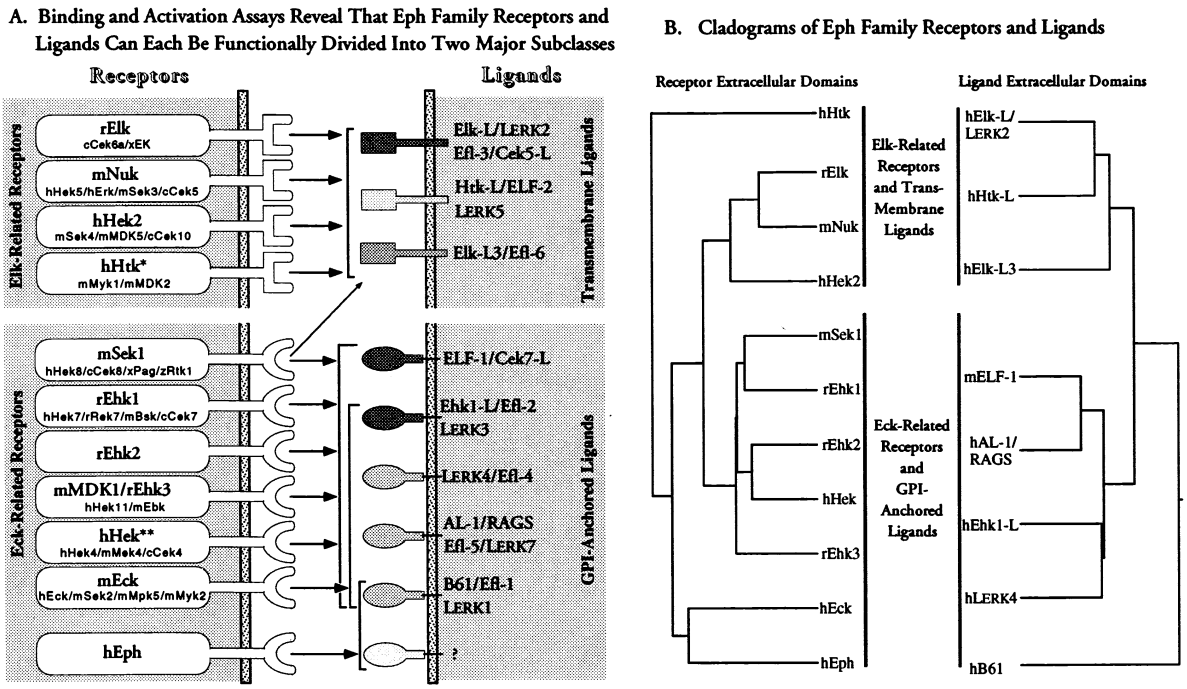

Figure 6. Groupings Made Based on Binding and Activation Assays Reflect the Relatedness of the Ectodomains of the Eph Receptors and Their Ligands

(A) Schematic summary of known receptor and ligand interactions. Arrows drawn from a receptor to a ligand group denote binding of that receptor to the ligands indicated by the brackets. The initial names of each receptor are provided in large bold type, with other names under which the receptors have been published indicated in smaller type; these names are prefixed with a lower case letter designating the species of origin for these receptors as follows: h, human; r, rat; m, mouse; c, chicken; x, Xenopus; z, zebrafish. Following the initial published names of the various ligands, additional published names of the various ligands are provided.

(B) Cladograms comparing the ectodomains of the Eph receptors and Eph ligands. Cladograms were generated by multiple amino acid sequence alignments using DNAStar Megalign program.

gands, which are free to access distant cells and thus may require exquisite receptor specificity to limit their actions, Eph family ligands can normally only act in membrane-anchored form (Davis et al., 1994). Thus, despite their ability to recognize many different receptors throughout the body, the actions of individual ligands may be restricted by simply limiting the distribution of the cells expressing the ligand, abrogating the need for exquisite receptor specificity. The evolutionary expansion of this family may have been driven not by the selection for new binding diversities, but rather by the selection for new distributions of old binding activities, or perhaps the association of new signaling capabilities with old binding activities. Regardless, the surprising lack of binding diversity in this large family raises issues concerning shared activities and redundancy; the fact that a given ligand maintains the ability to bind multiple receptors suggests that these interactions occur in vivo. Along these lines, it must also be considered that the subtle differences in the binding characteristics of members of the same subclass may prove to be functionally critical. For example, since this family has been implicated in the formation of gradients (Cheng et al., 1995; Drescher et al., 1995), it should be noted that "functional gradients" could, for example, either be formed by the graded distribution of an individual ligand, or by more uniform expression of several ligands in series, with each ligand having successively different affinities for a receptor recognizing the gradient; this may well be the case in the tectum, where AL-1/RAGS and ELF-1 display overlapping gradients of expression. In any case, our data demand that future functional analyses simultaneously consider the potential roles of all members of a particular subclass when examining a given biologic process in which one member of that subclass has been functionally implicated. The ability of any member of a given ligand or receptor subclass to mimic other members, at least pharmacologically, should also prove useful for addressing the roles of Eph family members in vivo.

The subclass designations we initially made based on in vitro binding and activation profiles were strikingly confirmed by staining whole embryos with receptor–bodies to define the in situ distribution of their ligands, or ligand–bodies to define the in situ distribution of their corresponding receptors. All receptors of a particular subclass identified similar ligand patterns in the embryo, while all ligands of a particular subclass detected similar receptor profiles. The distributions of receptors and ligands were in many cases consistent with emerging evidence that Eph family members may be involved in axonal outgrowth or guidance (Henkemeyer et al., 1994; Winslow et al., 1995; Drescher et al., 1995); for example, receptors recognizing the transmembrane class of ligands were noted on peripheral sensory ganglia and motor axons, and receptors for both classes of ligands were found in many regions of the developing brain. However, the most striking impression resulting from

the examination of receptor and ligand distributions was that expression of a receptor subclass was often quite complementary and reciprocal to that of its cognate ligand subclass, such that mutually exclusive receptor and ligand expression seems to subdivide much of the developing embryo into discrete domains. These subdivisions would not have been as readily apparent from distribution analysis of only individual members of a subclass, since each member accounts for only a portion of the composite pattern for that subclass.

Eph receptors seem to encounter their ligands primarily at the interfaces of the domains defined by the Eph subclasses, apparently demarcating precise boundaries throughout the developing embryo. These boundaries were noted in the developing brain and spinal cord, in the limb, in the branchial arches, in the somites, and elsewhere, implicating the Eph family in the formation, maintenance, or refinement of domains and boundaries in multiple embryonic structures, both within and outside of the developing nervous system. The strongest evidence for such a role for Eph family members has been obtained in the developing hindbrain. The binding studies presented here reveal that both the Eph ligands recently shown to be expressed in even-numbered rhombomeres (Htk-L/ELF-2 and Elk-L3; Bergemann et al., 1995; Gale et al., 1996) can bind to the Eph receptors (Sek1, Nuk/Sek3, and Htk/Sek4) previously shown to be expressed in odd-numbered rhombomeres. Dysregulation of these reciprocal expression domains, by uniformly expressing dominant negative versions of Sek1 throughout the hindbrains of developing Xenopus and zebrafish embryos, disrupts rhombomere specification (Xu et al., 1995), consistent with the notion that reciprocal expression of Eph receptors and ligands in adjacent rhombomeres is critical for normal segmentation in the hindbrain; earlier findings (Davis et al., 1994) that Eph family ligands can function only when presented in a membrane-bound form may be critical for their precise actions only at boundaries between distinct rhombomeres.

The developing limb presents an important example, with many analogies to the emerging hindbrain story, of how the dynamic boundaries and domains defined by Eph family expression can be compared with previous domains defined by fate-mapping studies and by the expression of other important regulatory molecules. Early in development, Eph-definable domains in the limb bud appeared to correlate with the three presumptive proximodistal subdivisions of the developing limb defined by fate-mapping studies, the autopod, the zeugopod, and the stylopod. Later in limb development, the Eph-definable domains in the distal limb precisely distinguished the developing cartilagenous digits from intervening regions that eventually regress and also marked the apical ectodermal ridge (at a stage in which it no longer has the ability induce outgrowth of the underlying mesenchyme). Interestingly, expression of a member of the Wnt gene family, Wnt-5a, seems to similarly distinguish the three proximodistal regions of the early limb and later specifically marks the developing digits (Dealy et al., 1993); the expression of particular Hox genes has been correlated with these areas of Wnt-5a expression (Dealy et al., 1993). Retinoic acid receptors (RARs) can also form proximodistal gradients in early limb, and different members of this transcription factor family seem to distinguish the developing cartilagenous digits from the interdigital necrotic zone in later limbs (Tabin, 1991); in fact, expression of Eph family members can be regulated by retinoic acid (Bouillet et al., 1995). Thus, in the developing limb, the domains and boundaries reciprocally marked by Eph receptors and ligands appear to correspond to those previously defined by fate mapping and by the expression of other regulatory molecules such as the Wnts, homeobox genes, or retinoic acid–binding transcription factors. These findings are very reminiscent of those in the hindbrain and suggest that the Eph family may be regulated by, and/or collaborate with, other signaling molecules, and in turn contribute to the process of shaping the developing limb, perhaps by refining or maintaining boundaries by providing repulsive or attractive cues, by regulating cell migration or axonal guidance into or out of particular domains, or perhaps by helping to specify domain-specific characteristics.

Our expression patterns also suggest major roles for the Eph family in the dorsoventral patterning of the spinal cord. The thin stripe of GPI-linked ligand expression that appears to bisect the developing neural tube dorsoventrally (Figure 5D) may correspond to a boundary, previously marked by the expression of the cell-adhesion molecule F-cadherin as well as by members of the paired box (Pax) transcription factor family, across which cell mixing is restricted (Espeseth et al., 1995). This possibility suggests that other dorsoventral divisions marked by reciprocal expression of Eph receptors and ligands in the developing neural tube may also have functional significance consistent with the proposed roles of the Eph family in regulating cell migration, mixing, or specification and that the Eph family may critically interplay with particular cell-adhesion molecules and transcription factors in mediating these functions.

The reciprocal expression patterns of Eph family receptors and ligands seem to mark boundaries corresponding to several previously noted functional compartments in the developing limb, hindbrain, and spinal cord. However, reciprocal Eph expression patterns we have noted also define many new boundaries in these and other structures that seem just as likely to be functionally important. We and others have noted intriguing relationships between the compartmentalization of Eph family members and an assortment of developmentally important transcription factors, such as members of the Hox, Krox, Pax, and RAR gene families. Since the actions of these transcription factors depends on cell-to-cell communication, they must specify signals at the cell surface that mediate their actions. The Eph family, which provides an example of cell-surface receptors and their ligands that are reciprocally compartmentalized in the developing embryo, become prime candidates to provide such cell surface signals, perhaps in collaboration with other important regulatory molecules such as members of the Wnt family and cell adhesion molecules.

Experimental Procedures

Construction and Preparation of Receptor– and Ligand–Fcs

Ehk1–Fc, Eck–Fc, and Elk–Fc receptor–bodies have previously been described (Davis et al., 1994), and Nuk–Fc, Sek1–Fc, Ehk2–Fc, Eph–

Fc, and Ehk3–Fc receptor–bodies and B61–Fc, Ehk1L–Fc, Elf-4/LERK4–Fc, AL1–Fc, HtkL–Fc, ELF1–Fc, and ElkL–Fc were similarly engineered and produced.

Cloning of Eph Family Ligands

The Efl-4 cDNA (subsequently found to be identical to LERK4) was originally cloned by an expression cloning strategy essentially as described (Davis et al., 1994), except that the cDNA library was constructed from the human osteosarcoma cell line 143B, which exhibited binding to several Eph family receptors including Ehk1, Ehk2, Ehk3, and Eck. An Efl-5 cDNA fragment originally amplified from mouse hindbrain cDNA using degenerate primers (A. F. et al., unpublished data) was used as a probe to isolate a full-length Efl-5 human coding region subsequently found to be identical to AL-1. ELF-1 and Htk-L coding regions were amplified from embryonic mouse cDNA sources based on published sequence information (GenBank accession numbers U14941 and L38847).

Cell Transfections and Binding Analysis

COS7 cells transient transfections were done using LipofectAMINE reagent and Opti-MEM I (GIBCO BRL) according to the instructions of the manufacturer. Binding to transfected cells was performed as previously described (Davis et al., 1994). Fc–fusion proteins were quantitated by ELISA using anti-human antibody (Jackson Immunoresearch) coated plates and an alkaline phosphatase–conjugated anti-human antibody (Promega) with the soluble substrate pNPP (Sigma).

Receptor Phosphorylation Assays

Receptor phosphorylation was determined as previously described (Davis et al., 1994) on cells that were 80%–90% confluent at the time of treatment and changed into serum-free media for 1–2 hr prior to stimulation. Cells were stimulated at 37°C for 30 min using ligand–bodies at 500 ng/ml and clustering antibody at a final concentration of 17 μg/ml (Jackson Immunoresearch, goat anti-human antibody).

Whole-Mount Embryo Staining

Embryos of the indicated stage were dissected in ice-cold PBS, blocked in blocking solution (BS: 10% goat serum, 2% BSA, 0.02% Na azide in PBS) for 1 hr, and then incubated with either COS cell supernatants (with 10% calf serum) containing ligand–Fc or receptor–Fc, or purified Fc fusions at a concentration of 5 μg/ml in BS. Embryos were then washed extensively with PBS and fixed with fresh 4% paraformaldehyde overnight. (Alternatively, to enhance visualization of surface staining, embryos were fixed briefly with −20°C methanol and subsequently rehydrated in PBS.) Fixed embryos were heat treated at 70°C for 60 min in PBS to kill endogenous phosphatases, reblocked in BS plus 0.1% Triton X-100 overnight, and then incubated with goat anti-human (alkaline phosphatase conjugated; Promega) at a 1:1000 dilution in BS. Embryos were then extensively washed in TBS plus 0.1% Triton X-100 and, following color development, embryos were postfixed in 4% paraformaldehyde for 3 hr to overnight and were subsequently equilibrated in 50% glycerol followed by 70% glycerol.

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