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Identification and Cloning of ELF-1, a Developmentally Expressed Ligand for the Mek4 and Sek Receptor Tyrosine Kinases

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Summary

Mek4 and Sek are tyrosine kinases with expression patterns in the mouse embryo that suggest important functions in early development. However, like all Eph family kinases, both were identified as orphan receptors without known ligands. We show that Mek4 and Sek soluble receptor-alkaline phosphatase fusion proteins can be used in a procedure termed RAP in situ to identify regions of ligand expression in the mouse embryo. Based on this spatial information, a cDNA expression library was prepared, and was screened with the fusion proteins to identify Eph ligand family-1 (ELF-1). In cell lines and embryos, ELF-1 is membrane bound by a phosphatidylinositol tail, a feature that may account for unique biological functions. Its sequence is homologous with B61, a ligand for the Eck kinase, defining a family of related ligands. The expression domains of ELF-1, Mek4 and Sek indicate potential roles in embryonic patterning.

Introduction

The embryonic development of vertebrates involves a complex program of cellular behavior that is coordinated by cell–cell communication. However, the specific molecular mechanisms of this cellular communication are, for the most part, not well understood. Peptide growth factors that are ligands for receptor tyrosine kinases are excellent candidates for intercellular signaling molecules with important developmental roles. These ligands are known to have potent effects on a wide variety of cell activities in vitro, including survival, proliferation, differentiation, adhesion, migration, and axon guidance. The powerful signaling effects of these molecules are further emphasized by the ability of both the ligands and the receptors, when activated by mutation or overexpression, to become potent oncogenes and cause drastic cellular transformation (reviewed by Cantley et al., 1991; Schlessinger and Ullrich, 1992; Fantl et al., 1993).

Specific developmental roles have been demonstrated for some growth factors or for their tyrosine kinase receptors. For example, the c-Kit receptor tyrosine kinase, encoded at the mouse *W* locus (Chabot et al., 1988; Geissler et al., 1988), and its ligand KL, encoded at the mouse *Sl* locus (Flanagan and Leder, 1990; Copeland et al., 1990; Huang et al., 1990; Zsebo et al., 1990), determine the proliferation, survival, or migration of primordial germ cells, hematopoietic stem cells, and neural crest progenitor cells. Other examples are the neurotrophins and their Trk family receptors, with highly specific functions in the developing mammalian nervous system (Snider, 1994) and the fibroblast growth factor receptor, implicated in Xenopus mesoderm induction (Amaya et al., 1991). In invertebrates, too, receptor tyrosine kinases and ligands such as sevenless, bride of sevenless, breathless, and torso are known to play key roles in processes that range from setting up the primary embryonic axes to specifying the fate of a single cell in the ommatidium (Shilo, 1992; Zipursky et al., 1992). Taken together, the emerging picture of the developmental function of ligands that act through tyrosine kinase receptors is striking in that these molecules play key roles at all stages of embryonic development and in a remarkable range of different types of patterning process.

The receptor tyrosine kinases can be divided into families based on structural homology and, in at least some cases, obvious shared functional characteristics (Fantl et al., 1993). The family with by far the largest number of known members is the Eph family. Since the description of the prototype, the Eph receptor, sequences have been reported for at least ten members of this family, not counting apparently orthologous receptors found in more than one species. Additional partial sequences and the rate at which members are still being reported suggest the family is even larger (Maisonpierre et al., 1993; Andres et al., 1994; Henkemeyer et al., 1994; Ruiz and Robertson, 1994; Xu et al., 1994; Zhou et al., 1994; see also references in Tuzi and Gullick, 1994). Remarkably, despite the large number of members in the Eph family, all of these molecules were identified as orphan receptors without known ligands.

Nevertheless, the expression patterns determined for some of the Eph family receptors have implied important roles for these molecules in early vertebrate development. In particular, their timing and pattern of expression during the phase of gastrulation and early organogenesis have suggested functions in important but poorly characterized cell–cell interactions involved in patterning the embryo at this stage (Gilardi-Hebenstreit et al., 1992; Nieto et al., 1992; Henkemeyer et al., 1994; Ruiz and Robertson, 1994; Xu et al., 1994). For example, Sek (which is called Sek-1 in Becker et al., 1994) shows a notable early expression in the two areas of the mouse embryo that show obvious segmentation, namely, the somites in the mesoderm and the rhombomeres of the hindbrain, hence the name Sek, for segmentally expressed kinase (Gilardi-Hebenstreit et al., 1992; Nieto et al., 1992). As in Drosophila, these segmental features of the mammalian embryo are implicated as important elements in establishing the body plan. The observation that Sek expression precedes the appearance of morphological segmentation suggests a role for Sek in forming these structures or in determining segment-specific cell properties such as lineage compartmentation or gene expression (Nieto et al., 1992). Observations such as this have been indicative of important and unique roles for Eph family kinases in development, but further prog-

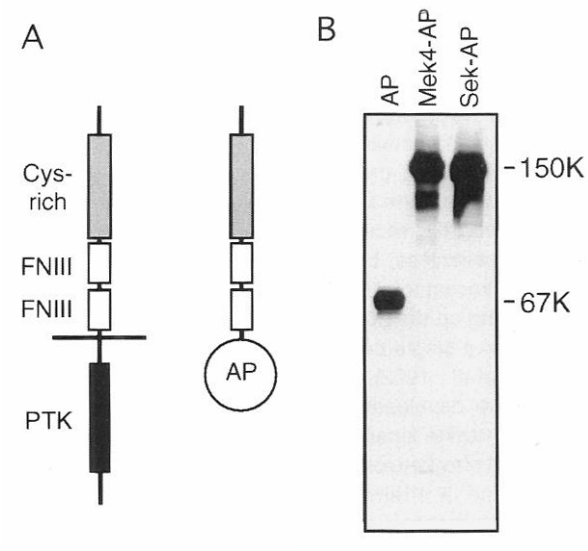


Figure 1. Mek4–AP and Sek–AP Soluble Receptor Affinity Reagents

(A) The Mek4 and Sek receptor tyrosine kinases are illustrated on the left. Like other members of the Eph family, each has a cysteine-rich domain with a characteristic spacing of cysteine residues (stippled box) followed by two fibronectin III (FNIII) motifs (open boxes), a single transmembrane domain, and an intracellular tyrosine kinase domain (PTK) (closed box). The diagram to the right illustrates the structure of the Mek4–AP and Sek–AP soluble receptor affinity reagents, each of which consists of the receptor extracellular domain fused to an AP tag.

(B) Expression of the Mek4–AP and Sek–AP fusion proteins and of unfused AP in the supernatants of transfected NIH 3T3 cells. Cells were metabolically labeled with [^{35}S]methionine, and then the supernatants were immunoprecipitated with a monoclonal antibody against human placental AP, separated on a 10% polyacrylamide gel, and autoradiographed.

ress in understanding their action has been severely limited by the lack of information on their ligands.

We have previously reported the use of a soluble receptor–alkaline phosphatase (AP) fusion protein to identify and characterize the ligand of the c-Kit receptor (Flanagan and Leder, 1990; Flanagan et al., 1991). Here we extend this approach and show that Mek4–AP and Sek–AP soluble receptor fusions can be used to localize sites of ligand binding directly in the mouse embryo, in a procedure we term RAP (for receptor affinity probe or receptor alkaline phosphatase) in situ. Using this information, the Mek4–AP and Sek–AP reagents were then employed to screen a cDNA expression library from a high expressing region of the embryo. The entire process of identifying and cloning a ligand by this procedure requires no purification or labeling of protein reagents and can be rapid and simple.

Eph ligand family 1 (ELF-1) was identified by this method, and we show here that it is a ligand that can bind to both Mek4–AP and Sek–AP. ELF-1 is an unusual tyrosine kinase ligand in being attached to the membrane through a glycosyl phosphatidylinositol (GPI) linkage. Its sequence is homologous to B61, a peptide that was originally identified as a cytokine-inducible cDNA of unknown function (Holzman et al., 1990) and that was recently shown to be a ligand that binds to the Eck receptor (Bartley et al., 1994). This homology defines a family of related ligands. The developmental expression of ELF-1 and its receptors Mek4 and Sek reveals complementary domains of expression that delineate potential roles for these molecules in patterning several developmental fields of the vertebrate embryo.

Results

Localization of Ligand(s) for Mek4 and Sek by RAP In Situ

To search for ligands for Mek4 and Sek, cDNA encoding each receptor extracellular domain was inserted into the vector *APtag-1* (Flanagan and Leder, 1990) to give a fusion with placental AP (Figure 1A). Figure 1B shows that Mek4–AP and Sek–AP are secreted proteins, and each was produced as a single major polypeptide with the expected apparent molecular mass of approximately 150 kDa. The AP provides a tag that binds to commercially available antibodies, allowing coimmunoprecipitation procedures. More significantly, it has an enzyme activity that can be traced quantitatively by simple chromogenic assays, without purification, radioactive labeling, or the use of secondary reagents. We find that detection using the enzyme activity of AP fusion proteins provides a sensitivity at least comparable to other approaches, such as the use of purified and ^{125}I-labeled reagents (Flanagan and Leder, 1990; Flanagan et al., 1991; M.-K. Chiang, A. Bergemann, H.-J. C., and J. G. F., unpublished data).

As the Mek4 and Sek receptors are known to be expressed during embryonic development, we decided to test whether the Mek4–AP and Sek–AP fusion proteins could be used to detect their ligand(s) directly in mouse embryos. Whole embryos were treated with receptor–AP fusion protein, washed, and then tested for bound AP activity of the fusion proteins using standard histochemical stains. A characteristic pattern of staining was detected when either Mek4–AP or Sek–AP was used to test embryos at day 9.5 of development (Figure 2). Similar staining was not seen in controls using unfused AP (Figure 2) or when other receptor–AP fusion proteins were used (data not shown). The patterns seen with Mek4–AP and Sek–AP were similar to one another, with the strongest reactivity in both cases seen in the region of the presumptive midbrain and anterior hindbrain (Figure 2). Reactivity was also seen in other areas, including the region of the somites, the branchial arches, a stripe down the dorsal face of the neural tube posterior to the hindbrain, and the limb buds (Figure 2). Strong and specific staining was also seen in embryos at days 8.5 and 10.5 of development (data not shown). It should be noted that the conditions used for these experiments were selected to minimize the possibility of losing or denaturing the ligand. The short incubation times and the absence of permeabilizing agents may favor staining of structures near the surface of the embryo. Also, as the embryos were not fixed prior to treatment with the receptor–AP reagents, the protocol used here may not be suitable for detecting freely diffusible ligands. However, in other experiments we find that the RAP in situ procedure can be modified to use fixed embryos, which may make it possible to detect soluble ligands also (unpublished data).

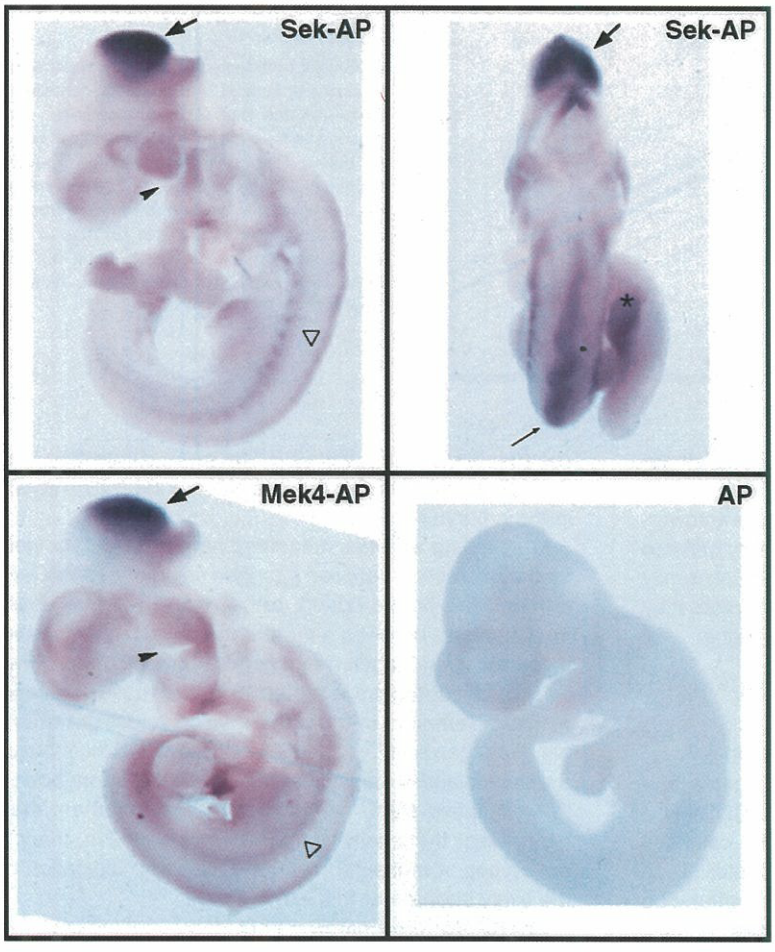


Figure 2. RAP In Situ of Mouse Embryos

Whole embryos at day 9.5 of development were treated with supernatants containing receptor fusion proteins or with unfused AP as a control and then were washed, fixed, and stained for bound AP activity. The thin roof of the fourth ventricle in the hindbrain was punctured to allow exchange of reagents with the lumen of the neural tube, resulting in a slight distortion in this region. Whole embryos are viewed laterally except for the upper right panel, which shows a dorsal view. Areas that appear to show specific staining include the following: midbrain and anterior hindbrain (arrow), branchial arches (arrowhead), dorsal face of the spinal cord (thin arrow), somites (open triangle), and limb buds (star).

Expression Cloning of ELF-1 from Mouse Embryos

The strong reactivity of the midbrain and anterior hindbrain in the RAP in situ of mouse embryos suggested that a ligand is expressed at high levels there. To clone a ligand cDNA, we excised this region from 80 embryos and used it to construct a cDNA expression library. The library was produced as pools of approximately 1000 clones and was screened by a sib selection procedure. DNA from each pool was transiently transfected into a plate of COS cells, and the cells were then tested by incubating with mixed Mek4–AP and Sek–AP supernatants, washing, and staining for AP activity in situ. After screening 36 pools, a positive pool was identified by the presence of intense AP staining coincident with the surfaces of several individual cells scattered around the plate (Figure 3A). This pool was subdivided and rescreened, and after a total of three rounds of screening (Figure 3B), DNA was prepared from single colonies and a positive clone, *E3-3*, was identified.

Nucleotide sequencing revealed a single long open reading frame in *E3-3* that could encode a polypeptide of 209 amino acids (Figure 4A). The deduced polypeptide begins with a methionine in a DNA sequence context consistent with a translation initiation site, followed by a typical hydrophobic signal sequence for peptide secretion (Figures 4A and 4B). This is followed by an amino acid sequence containing six cysteines and three potential N-linked glycosylation sites. The C-terminus ends with a stretch of 15 predominantly hydrophobic amino acids (Figures 4A and 4B), suggesting the presence of a signal for addition of a GPI tail that could anchor the polypeptide in the plasma membrane (Ferguson and Williams, 1988).

The polypeptide shown in Figure 4A appears to be a novel molecule that we named ELF-1. A search of the GenBank data base revealed one molecule with homology to ELF-1. This peptide, called B61, was identified as a cDNA of unknown function (Holzman et al., 1990) and was recently shown to be a ligand for the Eck receptor tyrosine kinase (Bartley et al., 1994). An alignment of ELF-1 and B61, shown in Figure 4C, gives an amino acid identity of 44% overall. The identity rises to 53%, with conservation of all four cysteines, over a core sequence from position 31 to 166 of ELF-1, excluding the N-terminal signal peptides and a C-terminal tail. The C-terminal tail shows poor conservation of primary sequence and could serve primarily as a linker for attachment to the membrane. The strong sequence conservation of ELF-1 and B61 is exceptional, though not unprecedented, for the ligands of receptor tyrosine kinases and defines a family of related ligands.

The Eph family receptors themselves show a high degree of sequence conservation, and in particular the close sequence similarity of their extracellular domains is unusual. However, on the phylogenetic tree of the family,

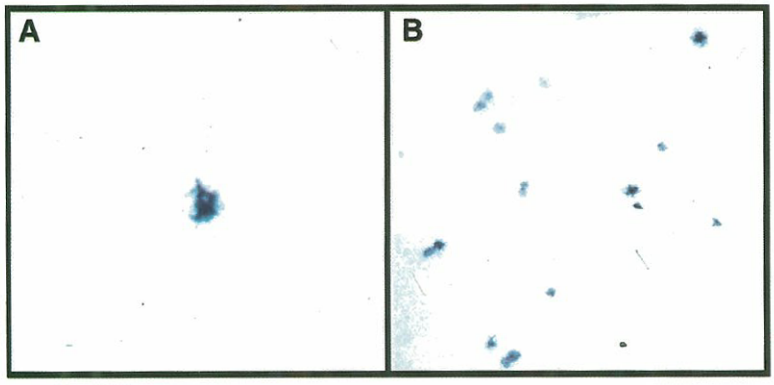


Figure 3. Screening a COS Cell Expression Library by RAP In Situ

COS cells transfected with library pools were treated with mixed supernatants containing Mek4–AP and Sek–AP fusion proteins, and then the cells were washed, fixed, and stained for bound AP activity. (A) A single positively stained cell in the primary round of screening. (B) A field of cells in the third round of screening. Magnification is lower in (B) than in (A). Unstained cells are not visible here, but the cells were nearly confluent at the time of staining.

the Eck receptor is distant from Mek4 and Sek, which are closely related to one another (Maisonpierre et al., 1993; Tuzi and Gullick, 1994). This makes it all the more noteworthy that ELF-1 and B61 are related. The similarity of these ligands for relatively divergent members of the Eph family suggests that other ligands for Eph family receptors may also have good conservation of primary sequence.

ELF-1 Is a Cell Surface Ligand for Mek4–AP and Sek–AP

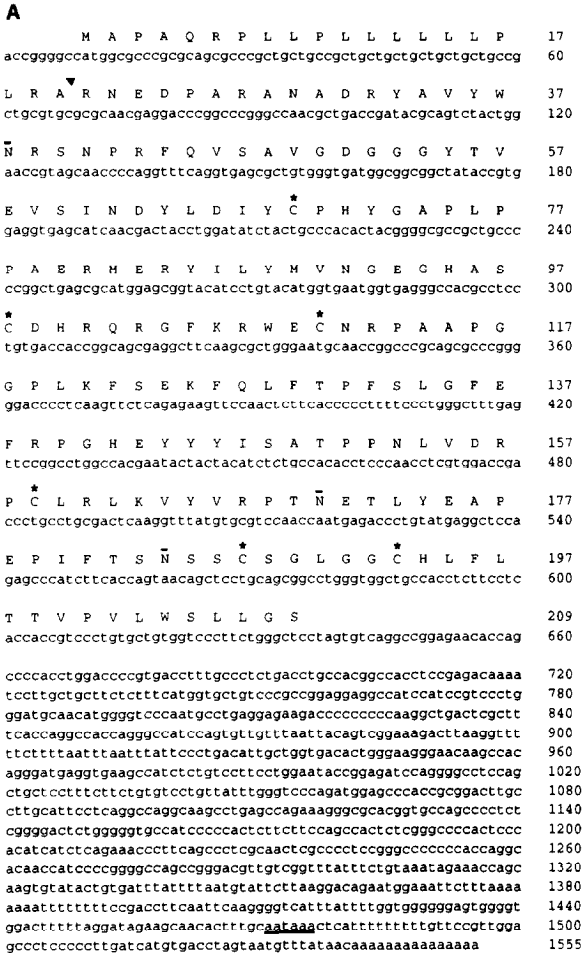
A quantitative analysis of the binding of Mek4–AP and Sek–AP to cell surface ELF-1 is shown in Figures 5A–5C. After transient transfection of ELF-1 into COS cells, binding of both Mek4–AP and Sek–AP to the cells can be detected, indicating that both of these receptor fusion proteins can bind to ELF-1 (Figure 5A). When saturating amounts of both Mek4–AP and Sek–AP are added simultaneously, the total AP binding is not additive, further confirming that they bind to the same ligand (Figure 5A). Scatchard analyses of the binding indicated that it is saturable and produced values for the dissociation constants (K_Ds) of approximately 10^{-9} M for Mek4–AP and approximately 10^{-8} M for Sek–AP (Figures 5B and 5C).

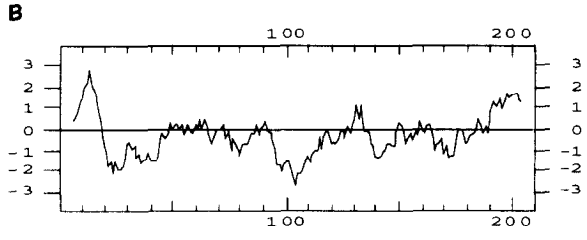
When endogenous expression of a cell surface ligand was tested in a panel of cell lines, some lines gave binding of both Mek4–AP and Sek–AP that was well above the AP control background, while others were negative (data not shown). One of the high binding lines, the rat liver stromal line BRL-3A, was analyzed further. As in the case of COS cells transfected with ELF-1, simultaneous treatment of BRL-3A cells with Mek4–AP and Sek–AP did not give additive binding of AP activity, indicating that the two fusion proteins bind to the same sites on the cell surface (Figure 5D). A Scatchard analysis of binding to BRL-3A cells (Figures 5E and 5F) indicated binding affinities for both Mek4–AP and Sek–AP that are similar to those found with ELF-1 in COS cells, consistent with ELF-1 being a ligand expressed on BRL-3A cells. The number of binding sites per BRL-3A cell is approximately 50,000. In situ staining was also used to examine the cell surface binding of Mek4–AP and Sek–AP to BRL-3A cells and indicated expression of the ligand over the whole cell surface (data not shown). It is interesting that RAP in situ staining of the mouse embryo midbrain–hindbrain region developed approximately two orders of magnitude more rapidly than comparable staining of the BRL-3A cells, suggesting that the ligand polypeptide is present at remarkably high levels in the embryo.

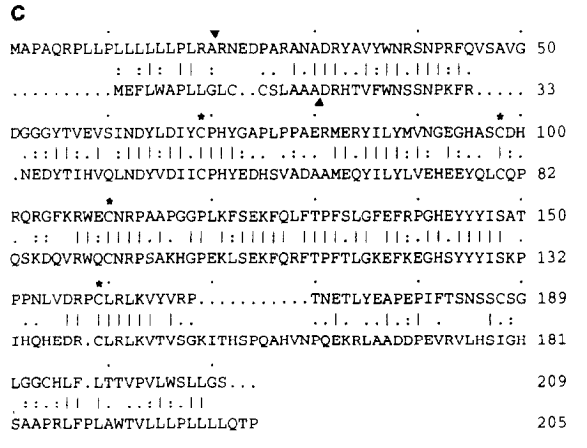
The binding affinities measured here were determined for the interaction between a tagged soluble receptor and a membrane-bound ligand, rather than the more usual measurement between a membrane-bound receptor and a soluble ligand labeled by chemical modification. However, other AP-tagged receptors or ligands have produced measured affinities in line with expected values (for example, Flanagan and Leder, 1990). Moreover, in the case of a receptor and a ligand that are both membrane bound in their native state, neither type of measurement may truly reflect the avidity of the interaction in vivo, though both types of measurement can give some indication of the likely strength of this interaction.

The K_D of approximately 10^{-9} M for the binding of ELF-1 to Mek4–AP is within the typical range of affinities for ligands binding to their cognate receptor tyrosine kinases, making it likely that this represents a genuine, biologically significant ligand–receptor interaction. The K_D estimate of 10^{-8} M for ELF-1 binding to Sek–AP is an affinity lower than many known receptor–ligand interactions, though not all. In this context it is worth noting that a similar (or slightly weaker) K_D of approximately 2×10^{-8} M to 3×10^{-8} M was reported for the interaction of the Eck receptor with its kinase-activating ligand B61 (Bartley et al., 1994).

Several additional factors are relevant in considering the biological significance of such an interaction. First, the avidity of the interaction between a receptor and a ligand that are both presented at the cell surface is likely to be greatly enhanced by the cooperative effect of highly multivalent ligand–receptor binding. Second, our results suggest that ELF-1 is present at very high localized concentrations in the embryo, another factor that would favor interaction with a receptor of moderate affinity. Additional support comes from our in situ studies of embryos. RNA hybridization in situ, described further below, indicated that in several regions of the embryo Mek4, Sek, or both are expressed in patterns that are complementary to the areas of ELF-1 expression, indicating likely interactions between these molecules during development. Furthermore, the RAP in situ with either Mek4–AP or Sek–AP (see Figure 2) detected a pattern of ligand distribution that was subsequently found to be strikingly similar to the pat-







tern of ELF-1 RNA expression (see Figure 7), indicating that ELF-1 is, at least, likely to be a major ligand for these receptors. However, it is also quite possible that ELF-1 may bind to other Eph family receptors or that Mek4 and Sek may have additional ligands.

ELF-1 Attachment to the Cell Surface Is Sensitive to Phosphatidylinositol-Specific Phospholipase C in Cell Lines and Embryos

The hydrophobic C-terminus of ELF-1 suggested a signal for linkage to a GPI tail that could account for the association of ELF-1 with cell surfaces. This possibility was tested by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). Mek4–AP and Sek–AP binding activity was found to be removed from the cell surface after PI-PLC treatment either of COS cells transfected with ELF-1 or of BRL-3A cells (Figure 6A). Binding activity of the ligand is not destroyed by this treatment, as coimmunoprecipitation experiments indicate that PI-PLC treatment also results in release of ligand polypeptide into the cell supernatant (Figure 6B). It therefore appears that cell surface ELF-1 in transfected COS cells and the cell surface ligand expressed endogenously in BRL-3A cells are attached to the cell membrane via a GPI linkage.

The effect of PI-PLC was also tested on embryos. In RAP in situ experiments, prior treatment of the embryos with PI-PLC resulted in a reduction of Mek4–AP or Sek–AP staining to background levels (Figures 6C and 6D). In the embryo, too, it therefore appears that the ligand(s) detected by Mek4–AP and Sek–AP is held in place by an association with cell surfaces via a GPI linkage. It is also possible that diffusible forms of the ligand(s) could exist as well.

In Situ Hybridization Analysis of ELF-1 RNA Expression in Mouse Embryos

If ELF-1 is indeed a ligand detected by the RAP in situ procedure, it would be expected that the pattern of ELF-1 RNA expression should overlap in at least some areas with the RAP in situ. An ELF-1 RNA probe was therefore used to examine mouse embryos by whole-mount in situ hybridization (Figure 7). The expression pattern detected by the ELF-1 RNA probe in day 9.5 embryos (Figures 7A and 7C) is remarkably similar to that seen in the RAP in situ experiments with Mek4–AP and Sek–AP (see Figure 2). The strongest expression was again seen in the region

Figure 4. Sequence of ELF-1

(A) Nucleotide sequence and deduced amino acid sequence of a cDNA clone encoding ELF-1. Cysteine residues are marked by asterisks, potential N-linked glycosylation sites are overlined, and a probable endpoint for the secretion signal sequence is indicated by a triangle. A polyadenylation signal in the 3′ untranslated sequence is underlined.

(B) Kyte–Doolittle hydrophobicity plot of the predicted ELF-1 polypeptide.

(C) Alignment of ELF-1 and B61. Identical amino acids are shown by a line, and conservative changes are indicated by dots between the two sequences. The putative endpoints for the secretion signal sequences are indicated by triangles. Conserved cysteine residues are marked with an asterisk. Sequences were aligned with the GAP program.

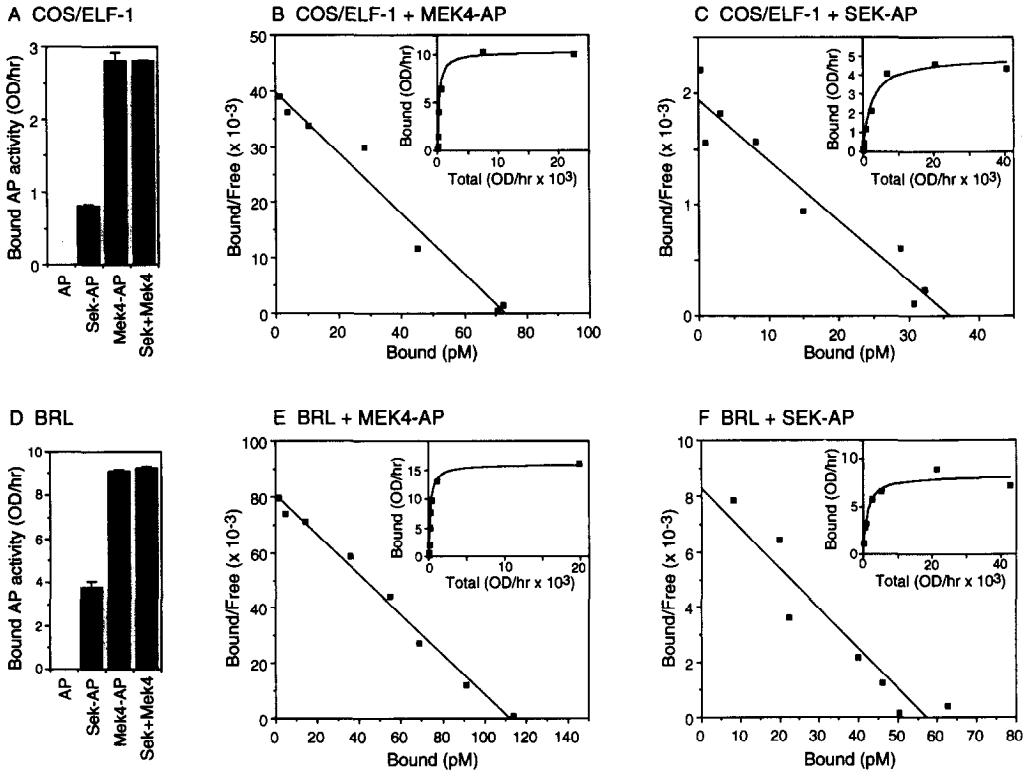


Figure 5. Cell Surface Binding of Mek4–AP and Sek–AP

Cells were treated with supernatants containing Mek4–AP, Sek–AP, or unfused AP as a control. The cells were then washed, lysed, and assayed colorimetrically for bound AP activity. (A and D) Cells were treated with saturating amounts of Mek4–AP or Sek–AP or with AP, each at 500 OD/hr/ml. Columns show the average of two binding determinations, and error bars indicate the difference between the two. (B, C, E, and F) Scatchard analyses of binding. (A–C) Binding to COS cells transfected with clone *E3-3*. (D–F) Endogenous ligand expression by the BRL-3A cell line. Binding characteristics calculated for the experiments shown are as follows. For Mek4–AP with ELF-1 in COS cells, 1.7×10^5 sites average per cell with $K_D = 1.8 \times 10^{-9}$ M; with BRL-3A cells, 5×10^4 sites per cell with $K_D = 1.3 \times 10^{-9}$ M. For Sek–AP with ELF-1 in COS cells, 1.0×10^5 sites average per cell with $K_D = 1.8 \times 10^{-8}$ M; with BRL-3A cells, 3×10^4 sites per cell with $K_D = 0.67 \times 10^{-8}$ M.

of the presumptive midbrain and anterior hindbrain (Figure 7A), and other areas of RNA expression were also similar to those seen in the RAP in situ (Figure 7A–7D). One apparent exception to this concordance was the dorsal face of the neural tube posterior to the hindbrain, where no obvious signal was seen with the ELF-1 RNA hybridization probe, although a prominent stripe of reactivity was seen with the Mek4–AP and Sek–AP reagents (see Figure 2). We do not presently know the reason for this difference, but an interesting possibility is that the RAP in situ technique may be detecting an additional ligand present in this region of the embryo.

To obtain further information on the potential roles of ELF-1, Mek4, and Sek in the embryo, we compared the RNA expression patterns for all three molecules by whole-mount in situ hybridization of embryos at days 8.5, 9.5, and 10.5 of development (Figure 7). We present our initial observations here. The Sek expression pattern has also been described more extensively by others (Nieto et al., 1992). Expression of ELF-1 and Sek RNA is prominent by the earliest timepoint analyzed, day 8.5 (Figures 7B and 7H). Mek4 RNA was less obvious at this time (data not shown) but is present at high levels by day 9.5 (Figure 7E). The regions of expression include the neural tube, branchial arches, somites, and limb buds. In the neural tube, the region of very high ELF-1 expression in the midbrain and anterior hindbrain (metencephalon) (Figures 7A and 7B) is flanked on one side by a region of high Mek4 and Sek expression near the forebrain–midbrain junction and on the other side by regions of Mek4 and Sek expression in the hindbrain (Figures 7E, 7H, and 7I). Expression of Sek in the hindbrain region is high in rhombomeres 3 and 5 and lower in rhombomeres 2, 4, and 6, as reported previously (Nieto et al., 1992). Expression of Mek4 appears to be highest in different rhombomeres, though a definite assignment will require further analysis. ELF-1 RNA expression in the somites, branchial arches, and limb buds appears mostly in a broad, diffuse pattern at this level of analysis (Figure 7A–7D). The Sek and Mek4 receptors are expressed in these same structures and within each are restricted to smaller subregions. Thus, in the somites, Mek4 is restricted to the dorsal part, the ventral part, or both (Figures 7E and 7G), while Sek is expressed in each new condensing somite in the day 8.5 embryo (Figure 7H) as well as in the dorsal part of each somite at later stages (Figures 7I and 7J). In the limb buds, Sek is expressed as a band at the distal end, becoming increasingly localized by day 10.5 (Figure 7J). Mek4 is expressed

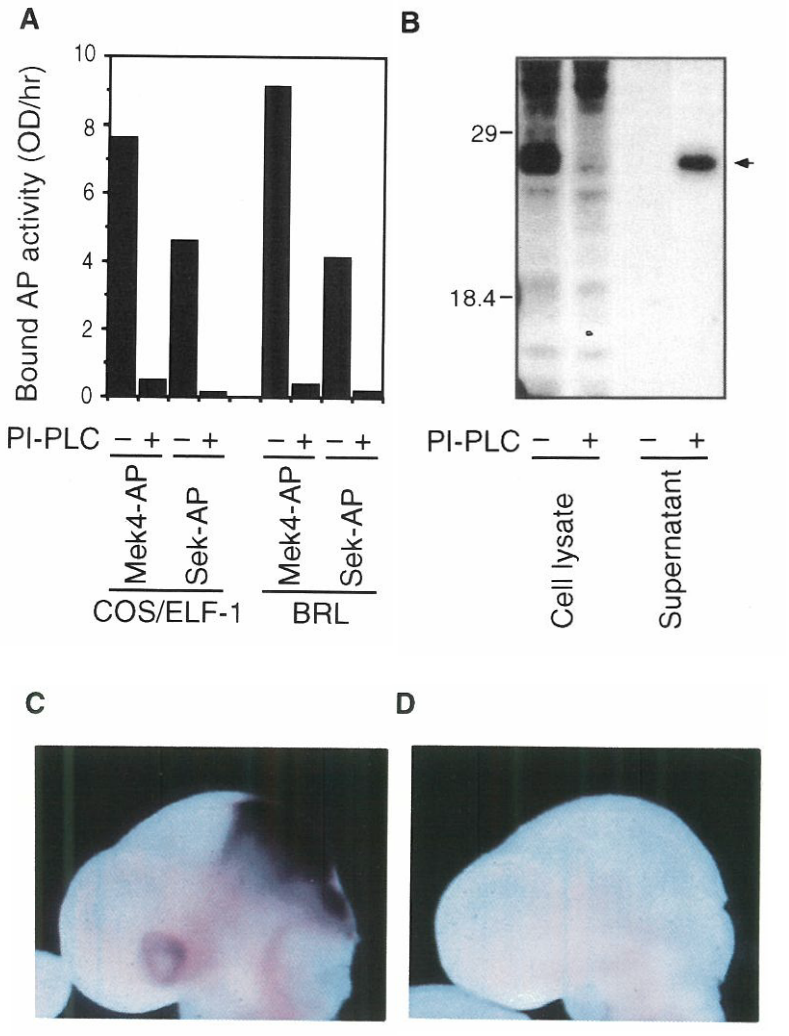


Figure 6. Effect of PI-PLC Treatment on Localization of Ligand for Mek4–AP and Sek–AP

Cells or embryos were preincubated with or without PI-PLC.

(A) Binding of Mek4–AP or Sek–AP to COS cells transfected with clone *E3-3* or to endogenous ligand on BRL-3A cells.

(B) Coimmunoprecipitation of ligand polypeptide from cell lysates or supernatants of BRL-3A cells. An arrow indicates the ligand polypeptide released into the supernatant by PI-PLC treatment. Sizes of molecular mass markers are shown on the left in kilodaltons.

(C and D) RAP in situ of Mek4–AP binding to day 9.5 mouse embryos preincubated without (C) or with (D) PI-PLC.

less prominently in the limb bud, in a posterior region near the junction of the limb buds with the flank, and in the lateral plate between the limb buds (Figures 7F and 7G). The expression in the branchial arches is complex and dynamic, but again Mek4 and Sek appear in localized subregions (Figures 7E, 7G, 7I, and 7J). A more comprehensive study will be required to determine the exact localization of expression in individual tissues and cell types. Also, as ELF-1 expression was seen at the earliest timepoint tested, it will be of interest to examine embryos at earlier stages of development.

Discussion

Identification and Cloning of a Novel Ligand from the Mouse Embryo by RAP In Situ

Growth factors that are ligands for receptor tyrosine kinases control a wide variety of cellular activities. Virtually all of these ligands that have been characterized are known to have important functions in development, physiology, or both and, in at least some cases, to be useful clinically. The existence of many additional hitherto unidentified ligands is implied by the discovery over the last few years of a large number of tyrosine kinases that appear by their structure to be cell surface receptors, yet have no known ligand. The rapid discovery of these orphan receptors has been possible mostly through techniques such as polymerase chain reaction that take advantage of the strong sequence conservation of the kinase catalytic domain.

Identification of the ligands for these orphan receptors has been more problematic, though it clearly represents a major opportunity to identify molecules that have important roles in controlling developmental and physiological processes. The ligands typically show low sequence similarity to one another, so methods based on sequence conservation are generally unfeasible. We have previously described an approach that makes use of the high affinity and specificity of ligand–receptor binding by using a soluble receptor–AP fusion protein to identify and characterize the ligand for the c-Kit receptor (Flanagan and Leder, 1990; Flanagan et al., 1991). Several other groups have by now also reported the use of soluble receptor reagents to identify ligands, and this type of approach appears to be widely useful (for example, Aruffo et al., 1990; Lyman et al., 1993; Bartley et al., 1994).

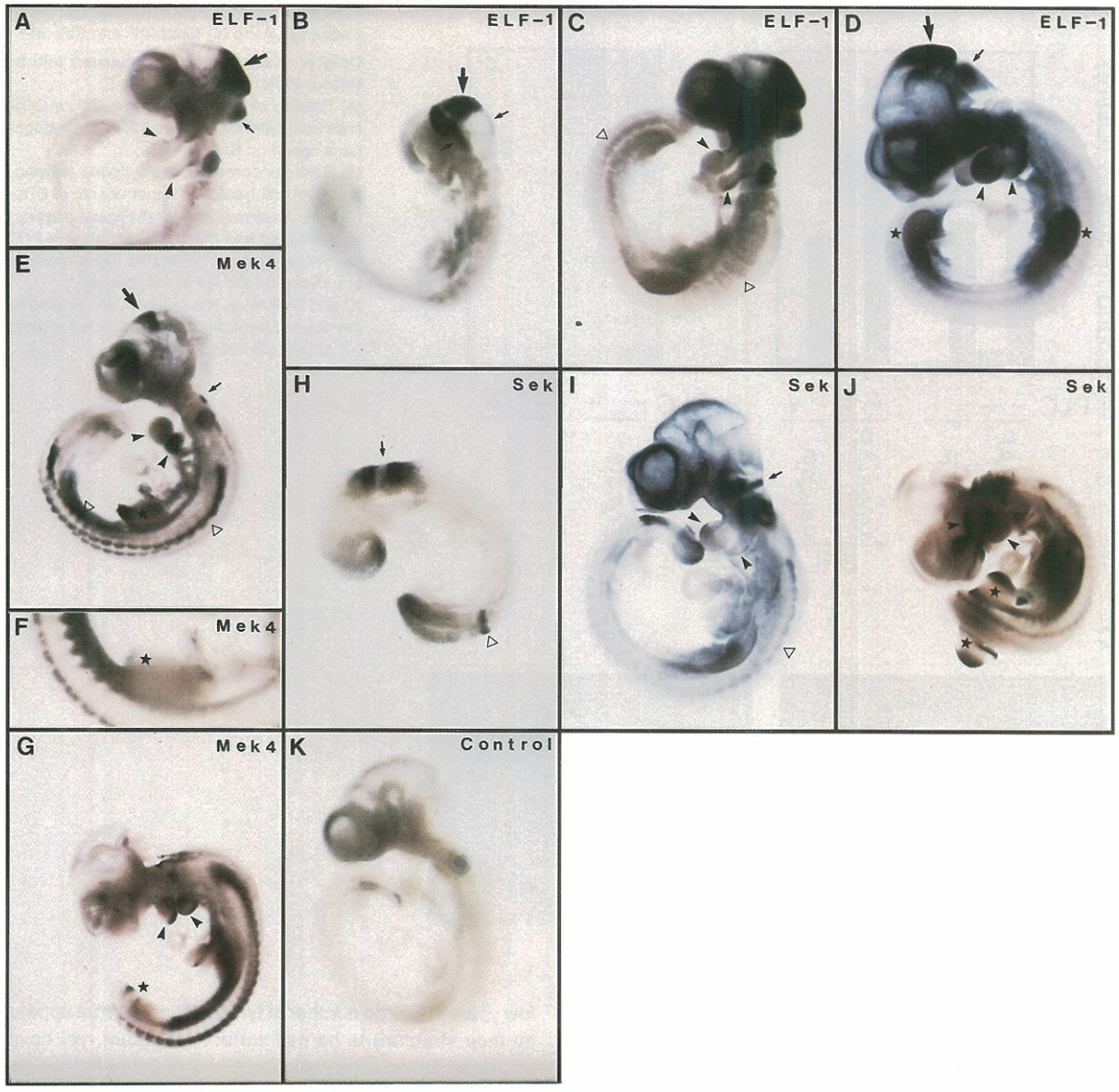


Figure 7. RNA Hybridization In Situ of ELF-1, Mek4, and Sek

Whole-mount preparations of mouse embryos are shown. (A–D) ELF-1 probe. (E–G) Mek4 probe. (H–J) Sek probe. (K) The control embryo shown here was treated with the Mek4 sense probe. (B and H) Day 8.5 embryos at the 10–12 somite stage. (A, C, E, F, I, and K) Day 9.5 embryos at the 25–29 somite stage. (D, G, and J) Day 10.5–11.0 embryos at the 35–44 somite stage. Structures that appear to show specific reactivity include midbrain (large arrow), hindbrain (small arrow), first and second branchial arches (arrowheads), somites (open triangle), and limb buds (star).

Such studies have generally been initiated by screening for ligand expression in candidate cell lines. However, we have been intrigued by the relatively high expression of known receptor tyrosine kinases and their ligands in whole embryos, at levels frequently comparable to or greater than those even in high expressing cell lines. Moreover, within the embryo, the expression of these molecules is usually found to be highly localized. These observations suggested that, to achieve a high enrichment of molecules of interest, it should be possible to make use of spatial and temporal biological information that is usually lost.

We show here that the soluble Mek4–AP and Sek–AP fusion proteins can be used to determine the expression pattern of a ligand directly in the developing mouse embryo by a technique called RAP in situ. This information was then combined with expression cloning to isolate a ligand, ELF-1, by dissecting out a region of high expression from embryos, preparing a cDNA library, and then screening it by essentially the same procedure used to characterize the embryo. This type of approach could presumably be applied to identify other novel ligands that bind to receptor tyrosine kinases or to other classes of orphan receptor.

In addition to identifying novel ligands, RAP in situ can

potentially provide other types of useful information. Constructing an organism involves not only controlling the behavior of individual cells, but also establishing pattern in three dimensions. Techniques that allow characterization of the spatial distribution of control molecules are therefore of central importance in understanding development. As RAP in situ detects natural receptor–ligand interactions, the information provided is qualitatively different from and complementary to both in situ RNA hybridization and in situ immunohistochemistry. Furthermore, this type of in situ analysis, based on the affinity of naturally interacting proteins, could undoubtedly be generalized from AP-tagged receptors to ligands or other molecules.

ELF-1, Mek4, and Sek in Embryonic Development

Intercellular signaling plays a key role throughout vertebrate development. A great deal of progress has been made, particularly in Xenopus, in understanding signals that mediate some of the earliest patterning events (Amaya et al., 1991; Jessell and Melton, 1992). However, little is known about signals that regulate many of the important events that unfold as gastrulation and early organogenesis proceed. Sek, Mek4, and some other members of the Eph family have been particularly intriguing in this regard because of their expression domains at this stage of development (Sajjadi et al., 1991; Nieto et al., 1992; Henkemeyer et al., 1994; Ruiz and Robertson, 1994). However, the lack of knowledge about the ligands for the Eph family receptors has been a major factor impeding further investigation of their biological roles.

The identification of ELF-1 has allowed us to initiate studies into its developmental function. In situ RNA hybridization, as well as RAP in situ, indicated that ELF-1 is expressed by day 8.5 of mouse development, soon after gastrulation. Earlier stages have not yet been examined, but will be of interest, particularly as a Sek homolog is expressed in the zebrafish from the onset of gastrulation (Xu et al., 1994). In several regions of the mouse embryo, we find that ELF-1 and its receptors Mek4 and Sek are expressed in apparently complementary patterns, indicating potential roles for their interaction.

In the developing neural folds and neural tube, the early expression of ELF-1, as well as the complementary expression of Sek and Mek4 in the forebrain, midbrain, and hindbrain, supports roles in early patterning events. In the hindbrain, it is intriguing that expression of Sek (Nieto et al., 1992) as well as ELF-1 and Mek4 appears to be correlated with rhombomere boundaries. Individual rhombomeres show a number of segment-specific cellular and molecular properties; for example, they can act as cell lineage compartments, they show differential expression of *Hox* genes, and they give rise to distinct streams of neural crest cells that emigrate into the branchial arches (Krumlauf, 1993). It is therefore plausible that ELF-1, Sek, and Mek4 may participate in defining at least some of these segmental properties. For example, the correlated expression of Sek and specific *Hox* genes in rhombomeres 3 and 5 suggests that Sek could be up- or downstream of the *Hox* pathway (Nieto et al., 1992; Krumlauf, 1993). Not all the areas of ELF-1, Mek4, and Sek expression in the hindbrain region appear to be immediately adjacent, so it is possible that interactions of these molecules with other receptors or ligands may also contribute to patterning here.

The expression patterns in the limb bud are also intriguing. In each limb bud, ELF-1 appears to be expressed in a diffuse central pattern, while Sek is expressed in a defined band at the distal end and Mek4 is expressed less strongly in a posterior proximal region. It is interesting that this expression shows some correlation with the structures at the distal tip and posterior margin that are known to play key roles in setting up the proximodistal and anteroposterior axes of this developmental field (Tabin, 1991). A similarly complementary pattern of ligand and receptor expression is seen in the branchial arches and somites and would be consistent with roles in either the formation or subsequent fate specification of each of these structures. Additional studies are now needed to assess further the precise roles of these molecules in patterning the embryo. Such studies should be greatly facilitated by the availability of cloned ELF-1.

Cell Surface ELF-1 and Spatial Patterning

The need to generate a complex three-dimensional pattern in development implies that key cellular communication molecules must be able to transmit accurate spatial information. This may explain why, although the first growth factors were identified as soluble molecules, it is increasingly becoming apparent that many, if not most, peptide growth factors can exist in forms that are not freely diffusible (Jessell and Melton, 1992; Bernfield et al., 1992; Massagué and Pandiella, 1993).

For several ligands of tyrosine kinases, this anchorage is mediated by the presence of a transmembrane domain. In the case of the Kit ligand/*Sl* factor, genetic evidence indicates that the presence of this transmembrane domain is essential for the molecule to fulfill its normal developmental function (Flanagan et al., 1991; Brannan et al., 1991). The precise biological roles of transmembrane anchorage are not clear, but may include tight localization of ligand activity and may also be related to the ability of these ligands to mediate cell–cell adhesion and promote cell migration (Flanagan et al., 1991; Massagué and Pandiella, 1993). Still other tyrosine kinase ligands are anchored by interactions with proteoglycans or other molecules in the extracellular or pericellular matrix. These interactions localize the ligands and can also have major effects on their biological activity (Bernfield et al., 1992; Jessell and Melton, 1992).

Like many other ligands for receptor tyrosine kinases, ELF-1 is presented at the cell surface. However, the mechanism of this anchorage is unusual in that it apparently involves a C-terminal GPI tail. This is seen for ELF-1 in cell lines, as well as in the embryo, where all reactivity in RAP in situ experiments with Mek4–AP and Sek–AP was removed by PI-PLC. As the related polypeptide B61 also shows cell surface anchorage that is sensitive to PI-PLC (Holzman et al., 1990; Bartley et al., 1994), the presence of a GPI linkage may emerge as a general feature of the Eph ligand family.

This linkage adds a novel dimension to the anchorage mechanisms of ligands for receptor tyrosine kinases and is particularly interesting in view of the proposed functions of the Eph family receptors. For example, the segment-specific cellular and molecular properties of the rhombomeres imply the existence of cell–cell interactions that are highly restricted spatially and that could be mediated by Eph family receptors, including Sek and Mek4. Also interesting is an immunohistochemical study of the Nuk receptor, which suggested possible functions in the guidance of neuronal migration, as well as in the pathfinding or fasciculation of the earliest axons of the nervous system (Henkemeyer et al., 1994). The presentation of ELF-1 and other Eph family ligands in a cell surface form with a GPI linkage could play an important part in determining the spatial specificity of these unique cell–cell interactions that play a critical role in early stages of patterning the developing vertebrate embryo.

ELF-1 and the Eph Ligand Family

In view of the biological importance of the ligands of receptor tyrosine kinases, it is remarkable that the largest family of receptor tyrosine kinases, the Eph family, should have had no known ligands. The lack of information on the ligands has presented a serious obstacle, but work on the receptors has nonetheless implied important functions for these ligands in several areas of biology. In addition to other aspects of their developmental function, the expression of Mek4, Sek, and numerous other Eph family receptors specifically in the nervous system of adult animals or in developing neurons has suggested that the ligands may act as neurotrophic factors (for example, Sajjadi et al., 1991; Gilardi-Hebenstreit et al., 1992; Maisonpierre et al., 1993; Henkemeyer et al., 1994; Zhou et al., 1994). The relevance of the Eph family receptors and ligands to cancer is suggested by observations such as the overexpression of HEK, a human homolog of Mek4, in certain hematopoietic tumors (Wicks et al., 1992). The downstream signaling pathways activated by ligand–receptor interaction will also be of interest, especially in view of the unusual kinase domain of the Eph family receptors, which shows similarity to both the cytoplasmic and the receptor tyrosine kinases. The identification of ELF-1 and the emergence of a family of related ligands should permit many new areas of research into the biological functions and the mechanisms of action of this novel class of signaling molecules.

Experimental Procedures

Construction and Expression of AP Fusion Proteins

To produce Mek4–AP and Sek–AP fusion constructs, cDNA sequences were amplified by polymerase chain reaction. For Mek4–AP the sequence from nucleotides 32 to 1708 (Sajjadi et al., 1991; GenBank accession number M68513) was amplified from mouse brain cDNA, and for Sek–AP the sequence from nucleotides 12 to 1698 (Gilardi-Hebenstreit et al., 1992; GenBank accession number X65138) was amplified from NIH 3T3 cells. Restriction sites at the ends of the amplification primers were cut with BamHI and inserted into the BglII site of the vector *APtag-1* (Flanagan and Leder, 1990) so that each receptor extracellular domain was fused through a 4 amino acid linker (Gly–Ser–Ser–Gly) to secreted human placental AP.

Each plasmid was linearized, and 2 μg (more can give lower expression) was transfected with 0.5 μg of *neo* selection plasmid and 20 μg of calf thymus carrier DNA by calcium phosphate precipitation into a 10 cm dish of NIH 3T3 cells. Cells were cloned in 96-well plates during G418 selection, and high expressers were selected by testing supernatants for AP activity in a 96-well plate reader or spectrophotometer as described (Flanagan and Leder, 1990) except that homoarginine was omitted from all AP assays here. AP activities are expressed here as OD units per hour (OD/hr); 1 pmol of AP fusion protein corresponds to approximately 30 OD/hr under the conditions of the assay. Clones expressing ≥1000 OD/hr of AP activity (about 5 μg of fusion protein) per milliliter of supernatant were used as a source of Mek4–AP and Sek–AP proteins and of control AP protein. Cells were grown to confluence, and then media conditioned for a further 3 days were centrifuged, 0.45 μm filtered, and stored at 4°C with 20 mM HEPES (pH 7.0) and 0.05% sodium azide.

RAP In Situ of Mouse Embryos

Embryos from Swiss–Webster mice were transferred to 1.5 ml microfuge tubes and rinsed once in HBHA buffer (Hank's balanced salt solution with 0.5 mg/ml BSA, 0.1% NaN_3, 20 mM HEPES [pH 7.0]) and then incubated in tissue culture supernatants containing receptor–AP fusion proteins (or containing AP as a control) for 75 min on a rotator at room temperature. They were then washed six times in HBHA buffer, treated for 2.5 min with an acetone–formaldehyde fixative (60% acetone, 3% formaldehyde, 20 mM HEPES [pH 7.0]), washed three times in HBS (150 mM NaCl, 20 mM HEPES [pH 7.0]), and then incubated in a 65°C water bath for 15 min, inactivating endogenous cellular phosphatases but not the characteristically heat-stable AP of the fusion proteins. After rinsing with AP buffer (100 mM Tris–HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$), the embryos were stained for 5–10 min in the same buffer containing 0.17 mg/ml BCIP and 0.33 mg/ml NBT.

Expression Library Construction and Screening

Eighty embryos at E9.5 from six Swiss–Webster mice were rinsed in PBS, and the region of the midbrain and hindbrain previously found to stain strongly by RAP in situ was cut from each embryo under a dissecting microscope. RNA was prepared by the single-step method, and after one round of oligo(dT) cellulose purification, the yield was 6.4 μg. Double-stranded cDNA was prepared (Invitrogen kit), inserted into the expression vector *CDM8* (Aruffo et al., 1990), and transfected into Escherichia coli. Pools of clones were plated on filters and then replica plated. DNA was prepared from one replica and the other was stored.

To screen the library, DNA of each pool was transiently transfected into a 10 cm dish of COS cells with lipofectamine (GIBCO BRL). After 48 hr, the cells, just at or before confluence, were washed once in HBHA and then incubated in an equal mixture of Mek4–AP and Sek–AP supernatants for 75 min at room temperature. Plates were then washed six times in HBHA, treated for 30 s with acetone–formaldehyde fixative, and washed twice in HBS. Uniform heating to inactivate endogenous cellular phosphatases is critical and was achieved by incubating plates containing 10 ml of HBS in a single layer on a flat shelf in a 65°C oven for 100 min. Plates were then rinsed with AP buffer and stained for 0.5–12 hr in the same buffer containing 0.17 mg/ml BCIP and 0.33 mg/ml NBT. Staining was monitored periodically against a white background under a dissecting microscope. After identification of a positive pool, the stored library filter was replica plated again, and rescreening was done with successively smaller areas.

Quantitative Cell Surface Binding, Coimmunoprecipitation, and PI-PLC Treatment

Quantitative cell surface binding assays and coimmunoprecipitations with Mek4–AP and Sek–AP were performed essentially as described previously for Kit–AP (Flanagan and Leder, 1990; Flanagan et al., 1991). In brief, for surface binding, cells in 10 cm or 6-well plates were washed with HBHA and then incubated at room temperature for 75 min with supernatants containing Mek4–AP, Sek–AP, or AP. For Scatchard analyses, the supernatants were concentrated in an ultrafiltration cell (Amicon) and dilutions were made with HBHA. The cells were then washed six times with HBHA, lysed, and assayed colorimetrically for bound AP activity. For coimmunoprecipitation, cells in 6-well plates were metabolically labeled with [^{35}S]methionine, and then the supernatants, concentrated to 200 μl on a Centricon-10 (Amicon), and cell

lysates in 200 μl of 1% Triton X-100, 10 mM Tris–HCl (pH 7), 1 mM PMSF were incubated for 75 min at room temperature with an equal volume of supernatant containing Mek4–AP. Labeled ligand polypeptides were then coimmunoprecipitated using a monoclonal antibody against human placental AP (Medix Biotech).

For experiments to assess GPI linkage, cells or embryos were pretreated in complete tissue culture medium for 2 hr at 37°C with 100–300 mU/ml of PI-PLC (Sigma).

In Situ RNA Hybridization

Whole-mount in situ hybridization of mouse embryos was performed as described (Wilkinson, 1992) except that posthybridization washes were three times each in solutions 1 and 3, without solution 2 or RNase. The ELF-1 antisense probe was a 1 kb fragment from a unique PstI site to the 3′ end of clone *E3-3*. The Sek antisense probe was a 0.8 kb fragment from a BsmI site at nucleotide 878 to the transmembrane sequence at 1698, and the sense probe was a 0.8 kb fragment from nucleotide 12 at the 5′ end to a HindIII site at 879. The Mek4 antisense probe was a 1.1 kb fragment from a BsmI site at nucleotide 564 to the transmembrane sequence at 1708, and the sense probe was a 0.8 kb fragment from nucleotide 32 at the 5′ end to a HindIII site at nucleotide 897.

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