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ELF-2, a New Member of the Eph Ligand Family, Is Segmentally Expressed in Mouse Embryos in the Region of the Hindbrain and Newly Forming Somites

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The Eph receptors are the largest known family of receptor tyrosine kinases and are notable for distinctive expression patterns in the nervous system and in early vertebrate development. However, all were identified as orphan receptors, and only recently have there been descriptions of a corresponding family of ligands. We describe here a new member of the Eph ligand family, designated ELF-2 (Eph ligand family 2). The cDNA sequence for mouse ELF-2 indicates that it is a transmembrane ligand. It shows closest homology to the other known transmembrane ligand in the family, ELK-L/LERK-2/Cek5-L, with 57% identity in the extracellular domain. There is also striking homology in the cytoplasmic domain, including complete identity of the last 33 amino acids, suggesting intracellular interactions. On cell surfaces, and in a cell-free system, ELF-2 binds to three closely related Eph family receptors, Elk, Cek10 (apparent ortholog of Sek-4 and HEK2), and Cek5 (apparent ortholog of Nuk/Sek-3), all with dissociation constants of approximately 1 nM. In situ hybridization of mouse embryos shows *ELF-2* RNA expression in a segmental pattern in the hindbrain region and the segmenting mesoderm. Comparable patterns have been described for Eph family receptors, including Sek-4 and Nuk/Sek-3, suggesting roles for ELF-2 in patterning these regions of the embryo.

Ligands that bind to receptor tyrosine kinases are known to have powerful effects on a wide variety of cellular activities in vitro, including proliferation, survival, adhesion, migration, differentiation, and axon guidance. In the context of the living organism, too, receptor tyrosine kinases and their ligands are known to have important functions in normal development and physiology, as well as in cancer and other diseases (8, 14, 37). The receptor tyrosine kinases can be divided into families based on structural homology and, in some cases, obvious similarities in functional properties. While several of these receptor families have been characterized extensively, the family that contains by far the largest number of known members, the Eph family, is not well understood. At least 11 Eph family receptors have been described, not counting apparent orthologs found in more than one species, and additional partial sequences and the rate at which new members are still being reported suggest that the family is even larger (11, 36, 37).

Remarkably, every member of the Eph receptor family was identified as an orphan receptor without a known ligand. The distinctive expression patterns of the receptors have nevertheless suggested that the ligands are likely to have interesting roles in cell-cell signaling. Almost all the receptors are expressed prominently in the developing or adult nervous system, suggesting roles in neuronal development or function (4, 9, 11, 18, 20, 24, 28, 30–33, 35, 40). Also, during embryogenesis, many of the receptors are expressed in highly restricted patterns at the stage of gastrulation and early organogenesis, suggesting functions in patterning the embryo. In particular, the early expression of several of the receptors in the segmental structures of the embryo, the rhombomeres and somites, has been striking. For example, in mice, Eph family receptors Sek, Mek4, Eck/Sek-2, Nuk/Sek-3, and Sek-4 are all expressed in the developing hindbrain, with each showing a characteristic rhombomere-specific pattern (4, 9, 18, 20, 30, 32). Sek, Sek-4, and Mek4 also show expression in the somites, with Sek and Sek-4 being expressed in a segment-specific manner in a wave down the embryo in the new somites as they form (4, 9, 30).

We recently described the identification and cloning of ELF-1 (Eph ligand family 1) as a ligand for the Mek4 and Sek receptors (9). Other ligands for Eph family receptors have also been described recently, and, like the receptors, these ligands form a family of molecules sharing close sequence homology. The currently known family members, in addition to ELF-1, are B61, originally cloned as a cytokine-inducible cDNA (21) and recently identified as a ligand for the Eck receptor (3); ELK-L/LERK-2/Cek5-L, identified as a ligand for the Elk and Cek5 receptors (5, 12, 34); EHK1-L/LERK-3, identified as a ligand for the Ehk1 and Hek receptors (12, 23); and LERK-4, identified as a ligand for the Hek receptor (23). All the ligands described so far exist in membrane-associated forms. Four of them are anchored by a GPI (glycosyl phosphatidylinositol) tail, and one of them, ELK-L/LERK-2/Cek5-L, is anchored by a transmembrane domain.

Here we describe a new member of the Eph ligand family, ELF-2. The sequence of ELF-2 indicates that it is a transmembrane molecule, displaying particularly close sequence homology to the other known transmembrane ligand, ELK-L/LERK-2/Cek5-L. On cell surfaces, and in a cell-free system, ELF-2 was found to bind at least three Eph family receptors, Elk, Cek10, and Cek5, with dissociation constants (K_d) in the nanomolar range. Also, ELF-2 binding to a chimeric Elk receptor was found to be capable of inducing intracellular signaling. In situ hybridization analysis of ELF-2 shows expression in the mouse embryo during early organogenesis, including segment-specific expression in the hindbrain region and in the organizing somites. This expression positions ELF-2 temporally and

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spatially to interact with Eph family receptors and suggests possible functions in patterning the embryo at this stage.

MATERIALS AND METHODS

Identification of clones encoding ELF-2. The ELF-1 amino acid sequence (9) was used to screen for homologs in the DBEST database by using the BLAST program (1), resulting in identification of a fragment of cDNA showing homology to a short stretch of ELF-1. This sequence, accession number L13819, had been obtained from a human 3-month-postnatal whole brain cDNA library. To perform further studies, we amplified this sequence from reverse-transcribed human brain total RNA (Clontech) by PCR, using two oligonucleotides, GG AAGCTTATCAAATTCACCATCAAGTTTCAAG and AATGTCCGGCGC TGTTGCCGTCTGTGCTAGAAC. The resulting 334-bp PCR product was purified by agarose gel electrophoresis and was then radioactively labeled and used to screen a mouse brain cDNA library by hybridization, with a low-stringency wash at 50°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Seven hybridizing clones were isolated. One of the clones represents mouse ELK-L/LERK-2/Cek5-L. Five of them (designated *E2.2* to *E2.6*) appear by restriction analysis and sequencing to be overlapping clones of a single cDNA sequence, which is shown in Fig. 1. Nucleotide residues 316 to 652 of this sequence show 94% identity with the human cDNA fragment whose sequence had been deposited in the expressed sequence tag database. The remaining clone, *E2.1*, contains an open reading frame covering the complete amino acid sequence but is missing nucleotide residues 413 to 505 (Fig. 1).

Binding and focus formation assays. To construct an ELF-2 expression plasmid, first plasmid *pELF2G* was constructed by PCR amplification of the ELF-2 open reading frame in *E2.1* by using the oligonucleotide primers GGGGATC CGGCATGCCATGGCCCGGTCC and GGCTCGAGTCCCTGCGTGCCCA AGAACAA. The resulting PCR product was cloned between the *Bam*HI and *Xho*I sites of *pcDNAI* (Invitrogen). To make a plasmid including the exon missing from *E2.1*, the 512-bp *Msc*I fragment of *pELF2G* was replaced with the 605-bp *Msc*I fragment of *E2.3* (corresponding to bases 305 to 909 of the sequence). The resulting clone, *pELF2J*, includes the entire open reading frame shown in Fig. 1.

To test for ligand binding to receptors on cell surfaces, the extracellular domain of ELF-2 was fused to a placental alkaline phosphatase (AP) tag. First, a new expression vector, *APtag-2*, was constructed by transferring the *Hin*dIII-to-*Xho*I fragment of *APtag-1* (16), which includes both the polylinker and the AP coding sequence, into *pcDNAI*, to allow efficient transient expression in COS cells. The ELF-2 extracellular domain was amplified by PCR, with *pELF2J* as the template, introducing an artificial *Hin*dIII site at the 5′ end (nucleotide 1 in Fig. 1) and an artificial *Bgl*II site at the end of the extracellular domain. This fragment was then inserted between the *Hin*dIII and *Bgl*II sites of *APtag-2* to produce plasmid *pELF2K*, encoding a fusion protein with Glu-225 of ELF-2 joined to AP through a 4-amino-acid linker (Arg-Ser-Ser-Gly). COS cells were transfected with *pELF2K* by using Lipofectamine (GibcoBRL), and the production of ELF2-AP protein was monitored by assaying supernatant for heat-stable AP activity. The production of NIH 3T3 cell lines stably producing mouse Elk and chicken Cek5, Cek9, and Cek10 extracellular domains fused to the intracellular domain of trkB is described in detail elsewhere (7). Quantitative assays of receptor-ligand interaction using the binding of soluble AP fusion proteins to cell surfaces were performed as described previously (9, 16). Binding is indicated as the AP activity bound to cells in a 3.5-cm-diameter tissue culture well.

Binding assays in a cell-free system were performed with receptors fused to an AP tag in combination with ligands fused to an immunoglobulin (Ig) Fc tag. The ELF-2 fusion with Ig (ELF2-Ig) was constructed by ligating the *Hin*dIII-*Bgl*II fragment of *pELF2K* to a gene encoding a human IgG1 Fc region (2) in the *pcDNA1* vector. As a control, we used ELF-1 similarly fused to an Ig (10a). The construction of plasmids encoding AP fusions of Elk, Cek5, Cek9, and Cek10 is described elsewhere (7). Supernatant was collected from COS cells transfected with plasmids encoding ELF2-Ig or ELF1-Ig, and the fusion proteins were bound to protein A-conjugated Sepharose beads (Pharmacia) by incubating 500 μl of supernatant with 500 μl of beads at room temperature for 1 h. The beads were then washed twice with HBHA (Hanks' balanced salt solution with 0.5 mg of bovine serum albumin per ml, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.0], and 0.05% sodium azide), and 15-μl aliquots were incubated at room temperature for 2 h with supernatants containing receptor-AP fusions, each at 0.5 nM. The beads were then washed five times with HBHA and were assayed colorimetrically for AP activity as described elsewhere (9, 16).

The use of an Elk-TrkB chimeric receptor, with the extracellular and transmembrane domains of Elk fused to the intracellular domain of TrkB, to assess signaling by LERK-2 has been described previously (7). Essentially the same protocol was used in this work to study signaling by ELF-2. Plasmid *pAS42* was used for expression of the Elk-TrkB chimera (7). For ELF-2 expression, the 1.2-kb *Bam*HI-*Xho*I fragment of *pELF2J*, containing the full coding region of ELF-2, was transferred into the expression vector *pMEXneo* to create *pRB56*. Plasmids were transfected into NIH 3T3 cells in 10-cm-diameter plates by the calcium phosphate method, and after 14 days the cells were treated with Giemsa stain and foci were counted. Consistent results were obtained from three separate transfection experiments.

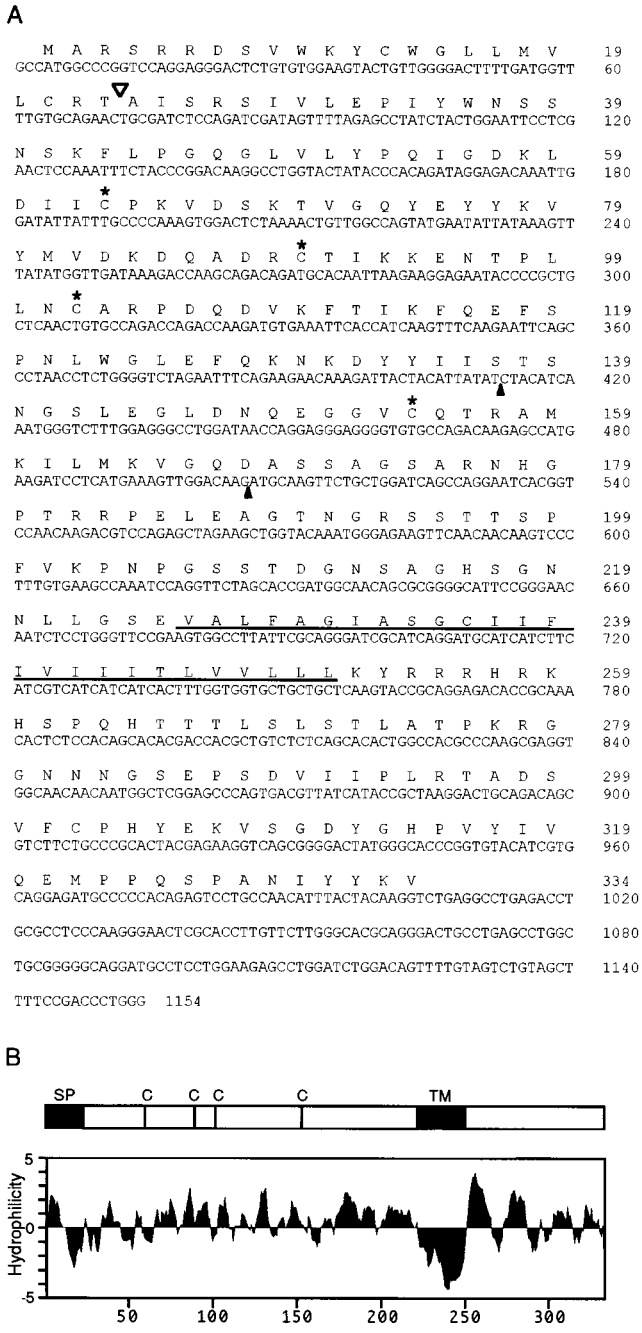

FIG. 1. Sequence of ELF-2. (A) *ELF-2* cDNA nucleotide sequence and deduced amino acid sequence. A triangle above the amino acid sequence indicates the predicted cleavage site for the secretion signal peptide. Underlining indicates the transmembrane domain. Asterisks indicate the four cysteine residues in the extracellular domain, which are conserved in other Eph family ligands. Arrowheads below the nucleotide sequence indicate the boundaries of the exon missing from clone *E2.1*. (B) Kyte-Doolittle hydrophilicity plot for predicted ELF-2 polypeptide. A diagram of the predicted structure of ELF-2 is shown above the plot, indicating the secretion signal peptide (SP), the transmembrane domain (TM), and the cysteines in the extracellular domain (C).

In situ analysis of *ELF-2* RNA expression. Analysis of *ELF-2* RNA expression was performed with three different probes. All three gave the same expression pattern. One, *pELF2E*, is a subclone of *E2.2* containing coding sequence extending from the 5′ end of clone *E2.2* at nucleotide 262 to a *Pst*I site at nucleotide

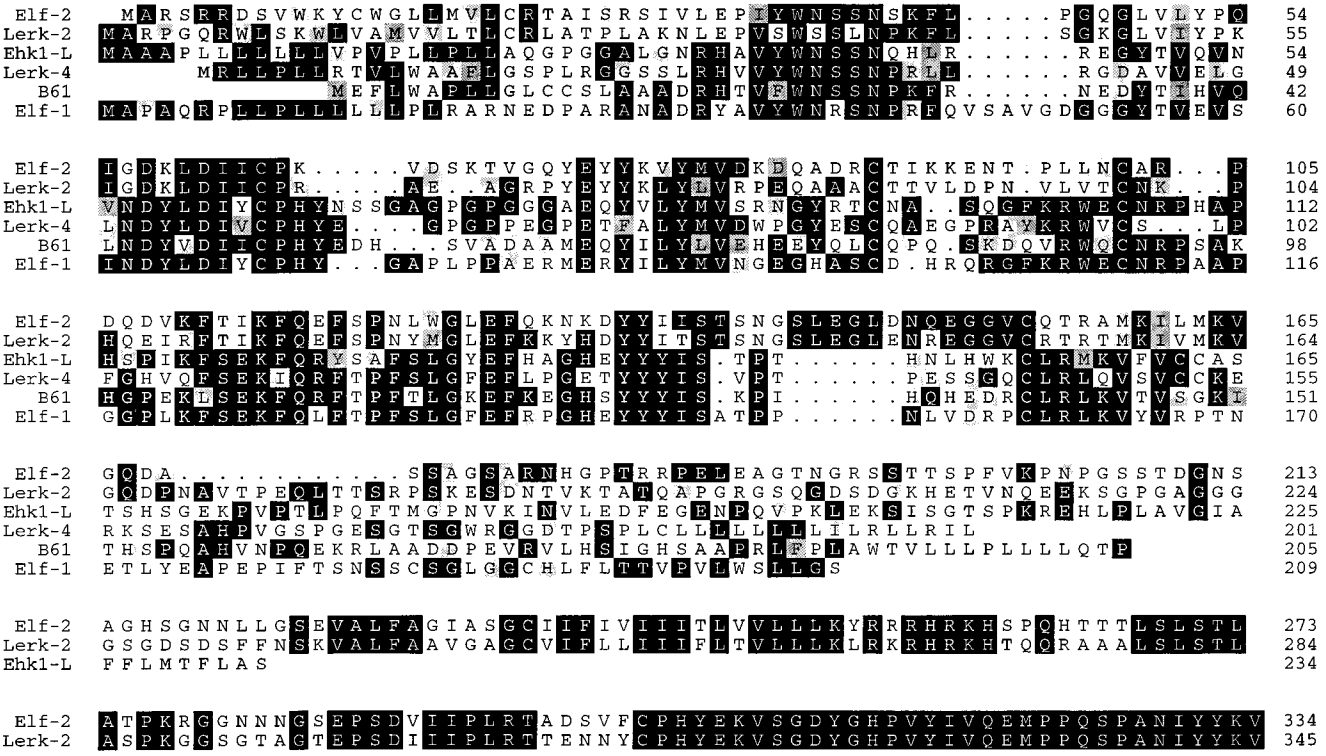

FIG. 2. Alignment of ELF-2 and other Eph ligand family members. Black boxes indicate majority amino acid residues in two or more sequences at that position. Grey boxes indicate residues that are conservative changes with respect to the residues in the black boxes. Sequences were aligned with the PILEUP program and displayed by using the PRETTYBOX program in the University of Wisconsin Genetics Computer Group package.

895. Two more subclones, *pELF2H* and *pELF2I*, were generated from the 3′ ends of clones *E2.1* and *E2.3*. These clones contain inserts of 700 and 500 bp, respectively, from the 3′ untranslated region of the cDNA. Although this region was not sequenced completely, partial sequencing and restriction mapping indicate that the region is shared by several independent *ELF-2* clones. All three subclones were made in the pBluescript SK vector and were cut at sites in the polylinker and used as templates for T3 and T7 RNA polymerases to produce sense and antisense probes for *ELF-2* RNA. Probes for *Sek* receptor RNA were produced as described previously (9). Labeling of probes with digoxigenin-UTP and in situ hybridization of whole-mount embryos were performed as described elsewhere (39), with modifications (9). For each developmental stage, consistent results were obtained by ELF-2 hybridization of at least five embryos from at least three different litters.

Nucleotide sequence accession number. The nucleotide sequence of *ELF-2* cDNA has been submitted to GenBank under accession number U30244.

RESULTS

Cloning and sequence analysis of ELF-2. After the cloning of ELF-1, a search of the computer databases for similar sequences revealed B61 as an obvious homolog (9). In addition, we noticed in the human chromosome 13 expressed sequence tag database a 337-nucleotide human cDNA fragment that, when translated, shares 41% identity with ELF-1 over a stretch of 39 amino acid residues. To investigate further whether this sequence might represent a new member of the Eph ligand family, this cDNA was isolated by PCR from a human brain cDNA library. The human cDNA PCR product was then used to screen a phage lambda cDNA library from newborn mouse brain. Seven hybridizing clones were identified. Nucleotide sequencing showed that one of these clones contains a murine cDNA for ELK-L/LERK-2/Cek5-L. The remaining six clones contained overlapping sequences that appeared to represent a novel cDNA. This cDNA sequence contains an open reading frame that could encode a 334-amino-acid polypeptide, which we named ELF-2 (Eph ligand family 2) (Fig. 1A). This open reading frame starts with a methionine codon in a nucleotide sequence context consistent with a translation initiation site (22), followed by a predicted signal peptide for secretion (38). The region between amino acid residues 226 and 251 is hydrophobic and is likely to represent a transmembrane domain (Fig. 1).

The ELF-2 deduced amino acid sequence shows close homology to sequences of other members of the Eph ligand family and includes the four cysteine residues that are conserved in all known members of the family (Fig. 2). The ELF-2 sequence shows particularly close homology to ELK-L/LERK-2/Cek5-L, the other transmembrane polypeptide in the family (Fig. 2 and 3). However, it is clear that ELF-2 represents a distinct new family member rather than an ortholog, since the ELK-L/LERK-2/Cek5-L sequence has been reported for mice as well as rats and humans (12, 17, 34). An alignment of ELF-2 with ELK-L/LERK-2/Cek5-L gives an overall amino acid identity of 60%, excluding the secretion signal sequence, and an identity of 57% in the extracellular domain. Over a core se-

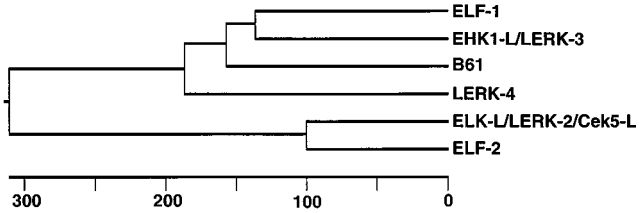

FIG. 3. Phylogenetic tree of the Eph ligand family. The tree was generated by using the MEGALIGN program (19).

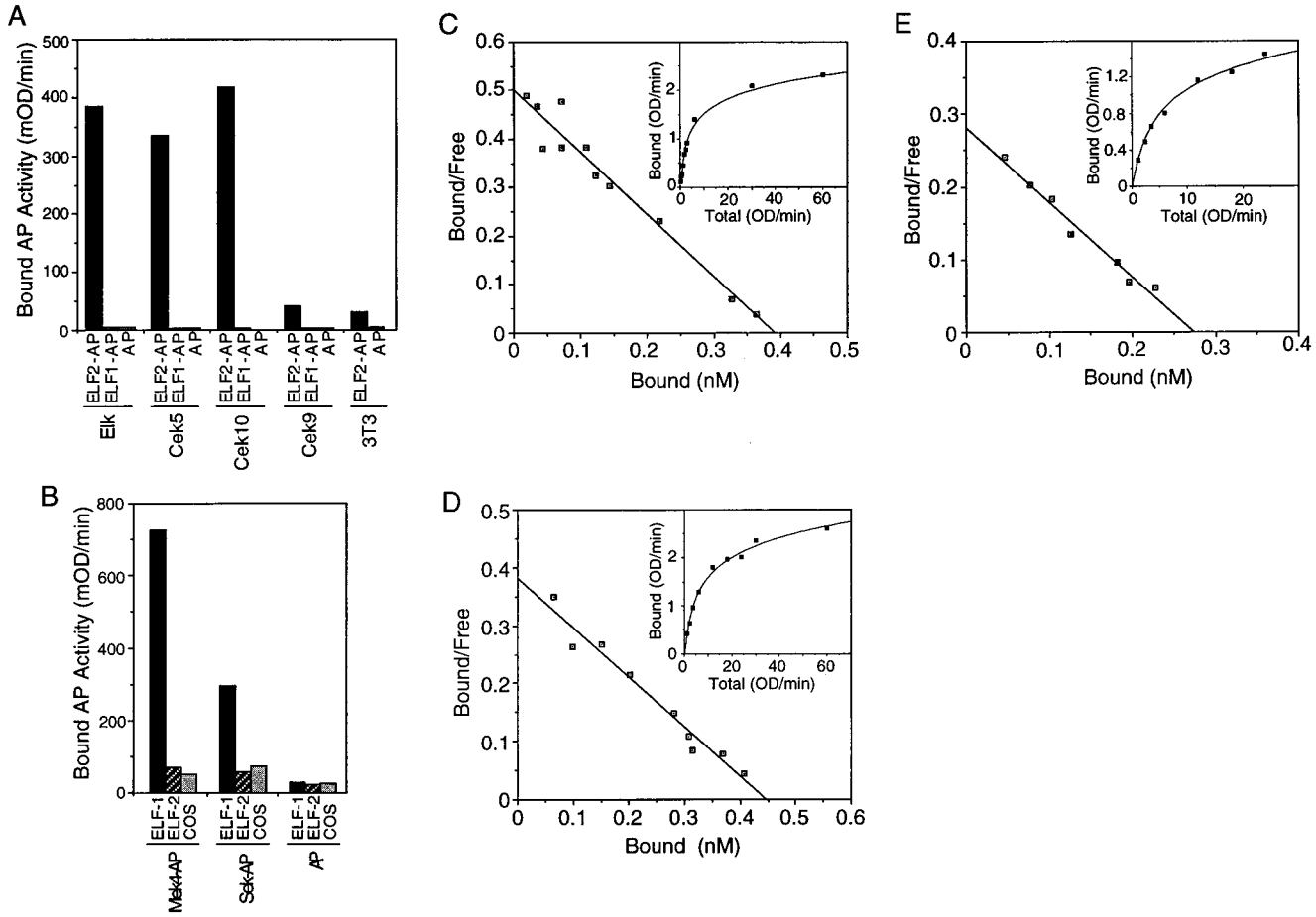

FIG. 4. Interactions of ELF-2 with Eph family receptors on cell surfaces. (A) Binding of ELF2-AP and ELF1-AP ligand fusion proteins and an unfused AP control, all at 3 nM, to receptors on NIH 3T3 cells stably transfected with constructs for Elk, Cek5, Cek9, or Cek10 or to untransfected NIH 3T3 cells. Each column indicates the average of two binding determinations, which differed by <10%. (B) Binding of Sek-AP and Mek4-AP soluble receptor fusion proteins and an unfused AP control, all at 30 nM, to ligands on COS cells transiently transfected with expression plasmids for cell surface ELF-2 or ELF-1 or to untransfected COS cells. (C, D, and E) Scatchard analyses of the binding of ELF2-AP to Cek10, Elk, and Cek5, respectively. Binding characteristics calculated for the experiments shown are as follows: for Cek10, 7.0×10^5 sites per cell with a K_d of 0.78×10^{-9} M; for Elk, 8.0×10^5 sites per cell with a K_d of 1.2×10^{-9} M; and for Cek5, 4.8×10^5 sites per cell with a K_d of 0.98×10^{-9} M. OD, optical density.

quence (residues 31 to 155 of ELF-2) that includes the four invariant cysteine residues and is well conserved across the family, ELF-2 again displays closest homology to ELK-L/LERK-2/Cek5-L. However, the 61% identity between the two molecules in this core region is not much higher than the overall identity, consistent with these being distinct ligands that could have different receptor-binding characteristics. A particularly striking aspect of the homology of ELF-2 with ELK-L/LERK-2/Cek5-L is the very close homology in their intracellular domains. The 83-amino-acid-residue intracellular domain displays an amino acid identity of 75%, with the last 33 amino acids being completely identical (Fig. 2).

One of the six ELF-2 clones, *E2.1*, contains a deletion of 93 nucleotides in comparison with the other clones (Fig. 1). This deletion corresponds exactly to the position of the third exon in the ELK-L/LERK-2/Cek5-L gene (17), implying that it represents an alternate splice form. The deletion maintains the same reading frame and would produce a peptide lacking amino acid residues 137 to 168, including one of the four invariant cysteine residues so far found in all members of the ligand family.

Characterization of the binding of ELF-2 to Eph family receptors. ELF-2 was tested for binding to six members of the Eph receptor family. Four of these receptors, Elk (25), Cek5, Cek9, and Cek10 (33), were expressed in stably transfected NIH 3T3 cells as fusion proteins consisting of the receptor extracellular domain fused to the intracellular domain of TrkB. To test for a binding interaction with these receptors, we created an ELF2-AP fusion protein, consisting of the ELF-2 extracellular domain fused to an AP tag. The receptor-expressing cells were then tested for binding of ELF2-AP (Fig. 4A). ELF2-AP bound to cell lines expressing the extracellular domains of Cek5, Cek10, and Elk, at levels well above those of controls; consistent results were obtained in multiple experiments, and typical binding data are shown in Fig. 4A. When tested for binding to a cell line expressing the Cek9 extracellular domain, ELF2-AP did not bind at levels detectably above the low-level endogenous binding shown by untransfected NIH 3T3 cells (Fig. 4A). To determine whether ELF-2 binds to Mek4 and Sek, COS cells were transiently transfected with an ELF-2 expression plasmid and were then tested for binding Mek4-AP and Sek-AP fusion proteins, consisting of the extracellular domains of Sek and Mek4 tagged with AP. No binding to ELF-2 was detected for either Mek4-AP or Sek-AP (Fig. 4B).

For Elk, Cek10, and Cek5, the three receptors that showed evidence of ELF-2 binding, Scatchard analyses were per-

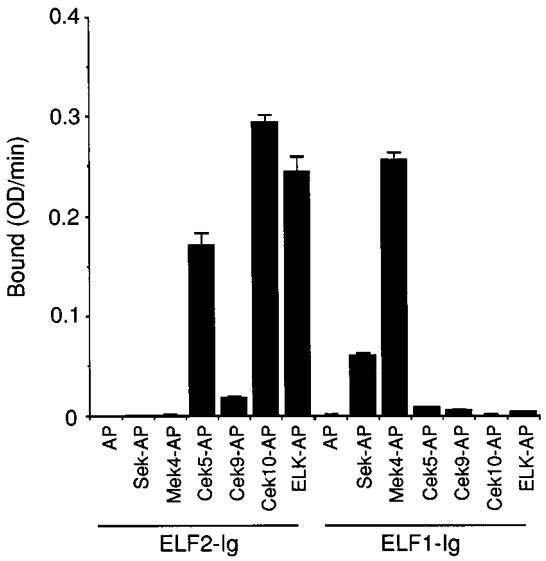

FIG. 5. Interactions of ELF-2 with Eph family receptors in a cell-free system. An ELF2-Ig fusion and an ELF1-Ig fusion used as a control were immobilized on protein A beads. The beads were then treated with receptor-AP fusion proteins, each at 0.5 nM, and then were washed and tested for bound AP activity. Control supernatants without ligand-Ig fusions showed no detectable binding with any of the receptors. Each binding assay was performed in triplicate; column heights show the mean, and error bars show the standard deviation. OD, optical density.

formed to determine the affinity of binding. For all three receptors, the binding interaction showed a reasonably high affinity, consistent with a biologically significant ligand-receptor interaction. In each case, the apparent K_d was approximately 10^{-9} M (Fig. 4C–E).

To establish that the interaction between ELF-2 and its receptors was a direct one, we also performed binding assays with a cell-free system. In this assay, ligands fused to an Ig Fc tag were immobilized on protein A beads and then were tested for their ability to bind receptor-AP fusions. The results indicate a direct interaction of ELF-2 with Elk, Cek10, and Cek5 but not with Mek4 or Sek (Fig. 5). Prominent binding to Cek9 was not observed, though the binding appeared to be slightly above background levels (Fig. 5), suggesting the possibility of a weak interaction. In contrast to ELF-2, the ELF-1 control bound only to Mek4 and Sek (Fig. 5). The results obtained with the cell-free system appear fully consistent with results from the cell surface binding studies.

ELF-2 induction of Elk-mediated signaling. To test whether receptor binding by ELF-2 can result in downstream signal transduction, we used a focus formation assay that was developed previously to demonstrate signaling in response to ELK-L/LERK-2/Cek5-L (7). Because proliferative and transforming effects of Eph receptors may be weak, we used for this assay a chimeric receptor with the extracellular domain of Elk fused to the cytoplasmic domain of TrkB (7). Davis et al. also described a similar approach, using Elk fused to a fibroblast growth factor receptor, to demonstrate a proliferative response to Eph family ligands (12). In this study, we transfected NIH 3T3 cells with plasmids encoding full-length ELF-2, Elk-TrkB, or both. The results show strong induction of focus formation by ELF-2, dependent on the presence of the Elk-TrkB receptor (Fig. 6).

In situ hybridization analysis of ELF-2 expression in mouse embryos. Studies of the Eph family receptors have shown very distinctive expression patterns during early organogenesis in vertebrates. We were therefore interested to test the temporal

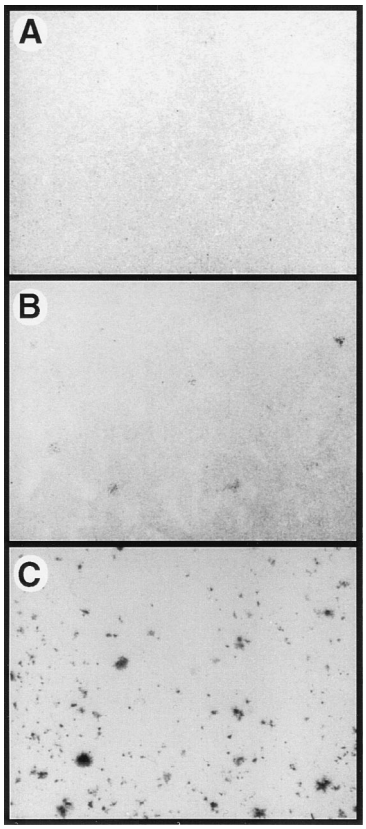

FIG. 6. Signal transduction by ELF-2, with an Elk-TrkB chimeric receptor. NIH 3T3 cells were transfected with (A) 1 μg of ELF-2 expression plasmid alone, (B) 0.5 μg of plasmid encoding the Elk-TrkB chimeric receptor, or (C) 1 μg of ELF-2 plasmid together with 0.5 μg of Elk-TrkB plasmid. Numbers of foci in this experiment on duplicate plates were as follows: ELF-2 together with Elk-TrkB, 292 and 284 foci; Elk-TrkB only, 32 and 32 foci; ELF-2 only, 0 foci. Foci were also larger following cotransfection of Elk-TrkB with ELF-2.

and spatial expression patterns of ELF-2 in the embryo. In situ hybridization analysis of *ELF-2* RNA in whole-mount mouse embryos was performed at days 8.5, 9.5, and 10.5 of development (Fig. 7A, C, E, and G). For comparison, in situ analysis was also performed for *Sek* receptor RNA (Fig. 7B, D, and F). At day 8.5 of development, soon after the onset of organogenesis, *ELF-2* expression is seen in the hindbrain and the branchial arches and as two intense bands in the region of somite formation at the boundary between somites and the presomitic mesoderm (Fig. 7H). Weaker staining is apparent in the segmented mesoderm and within restricted regions of the forebrain. At this stage, the staining in the region of the newly formed somites is similar to that of *Sek* (Fig. 7B), previously reported to be expressed in a wave down the embryo in association with each newly forming somite (30). In particular, *Sek* expression is seen prominently in two stripes: one in the somite currently condensing and another in the anterior portion of the most recently formed somite. *ELF-2* expression is similarly seen most prominently in a pair of bands. The posterior band is wider and is in or near the somite undergoing condensation, while the more anterior band is narrower, although often more intense, and is in or near the most recently formed somite. However, *ELF-2* differs from *Sek* in its lack of expression in more posterior regions of the presomitic mesoderm near the caudal end of the embryo (Fig. 7A).

ELF-2 expression is also seen in the hindbrain, with the

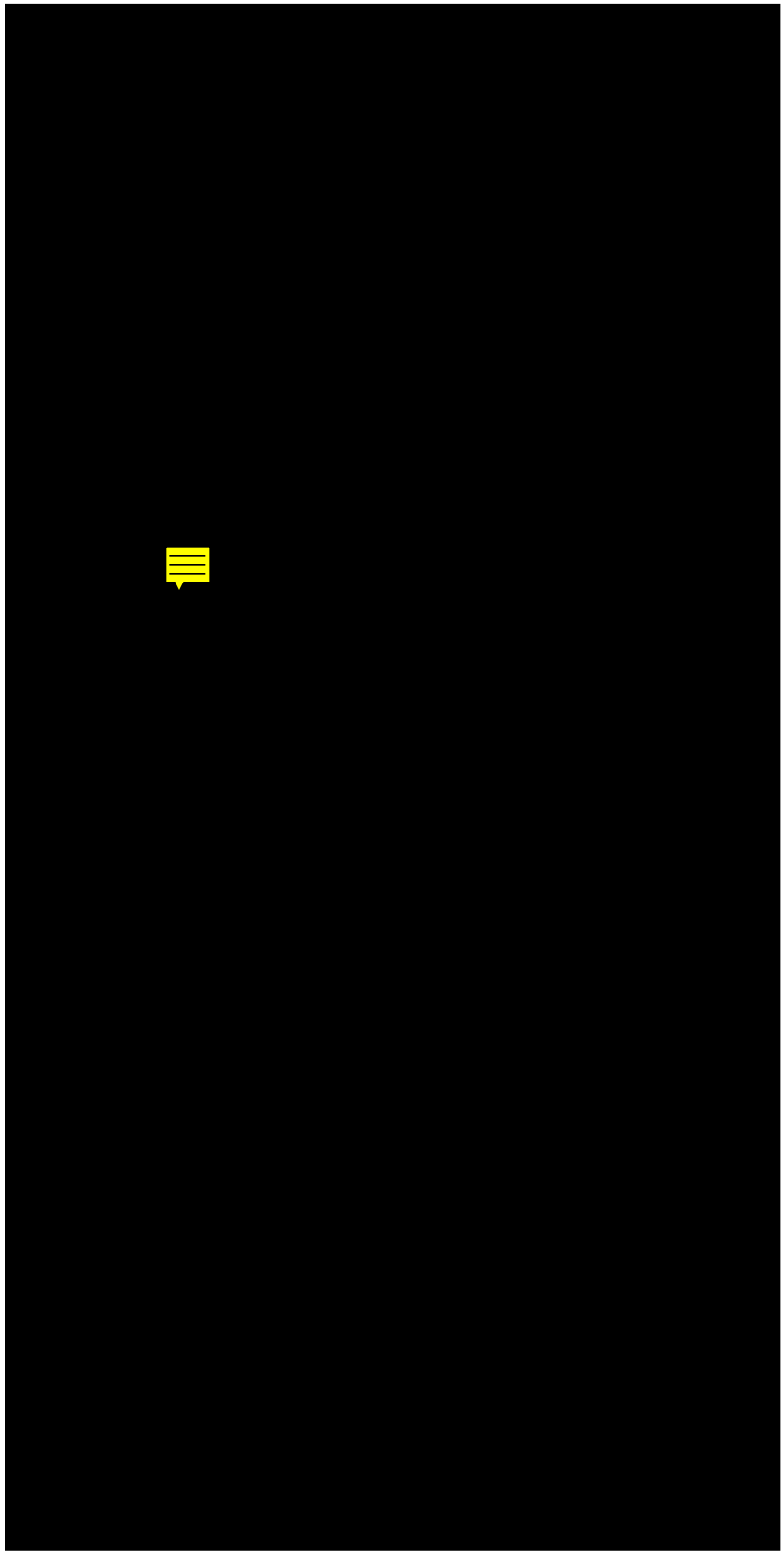

FIG. 7. In situ RNA hybridization of ELF-2 in mouse embryos. Whole-mount preparations of embryos are shown. (A, C, E, and G) ELF-2 probe; (B, D, and F) Sek probe for comparison; (H) ELF-2 sense-strand control probe. (A and B) Day 8.5 embryo at the 10- to 12-somite stage. (C, D, and H) Day 9.5 embryos at the 25- to 29-somite stage. (E, F, and G) Day 10.5 to 11 embryos at the 35- to 44-somite stage. Structures that appear to show specific reactivity include the hindbrain (arrowheads), forming somites (open triangles), limb buds (stars), branchial arches (short arrows), and forebrain (long arrows).

strongest expression being in the anterior hindbrain. Like several of the Eph family receptors, this ligand shows a segment-specific pattern in the hindbrain. On the basis of the relationship between *ELF-2* expression and the position of the otic vesicle, as well as a comparison with the *Sek* expression pattern, the expression of *ELF-2* at this stage was strongest at the level of rhombomeres 1, 2, 4, and 6 and was weak or absent at the level of rhombomeres 3 and 5 (Fig. 7A). When viewed dorsally, *ELF-2* expression is not seen at the midline but rather is confined to the dorsal or lateral region of the open neural folds.

In embryos at day 9.5 of development, *ELF-2* expression continues in all the regions noted above for day 8.5 embryos (Fig. 7C). Although less obviously segmented, the staining of the hindbrain remains strongest at the anterior end. The two bands of expression near the border between somites and the presomitic mesoderm remain clearly visible, with the anterior band still being generally narrower and more intense, and weaker expression can now also be seen in additional bands in more anterior somites of the segmented mesoderm. Strong expression remains in the branchial arches, while weaker expression is visible in the midbrain, particularly toward its anterior end, and in the forebrain.

At day 10.5 of development, *ELF-2* is most strongly expressed as bands in the region of somitogenesis, with the same pattern in this region as that described for the day 9.5 embryos (Fig. 7E). Compared with that at earlier stages, staining of the hindbrain is reduced by day 10.5. Expression is apparent in the branchial arches, in the forebrain, and near the optic cup and also in the limb bud, in a band adjacent to the prominent distal band of Sek expression (Fig. 7G).

DISCUSSION

All the members of the Eph family of tyrosine kinases were initially identified as orphan receptors, and none of their ligands were known until the recent identification of the first few members of a corresponding ligand family. We report here the isolation of a cDNA encoding a new member of the Eph ligand family. The molecule encoded by this cDNA, designated ELF-2, shows obvious homology to all five members of the Eph ligand family so far reported and is most closely related in primary sequence to ELK-L/LERK-2/Cek5-L.

Every member of the Eph ligand family so far identified contains a membrane anchorage domain. Three of the members, ELF-1, B61, and EHK1-L/LERK-3, are anchored by a GPI tail, a feature not so far identified in any other ligands that bind to receptor tyrosine kinases. In contrast, ELF-2 and its close relative ELK-L/LERK-2/Cek5-L have a transmembrane domain. Transmembrane domains have been found in a number of other ligands, including kit ligand (KL), and several members of the epidermal growth factor (EGF) family, such as EGF and transforming growth factor-α (TGF-α) (29). In KL, genetic evidence indicates that the presence of the transmembrane domain is essential for the normal functioning of the molecule in development (15). In general, it is likely that membrane anchorage of these ligands may be important in ensuring a tight localization of signaling, and this may be particularly true of the Eph ligand family, at least two members of which (EHK1-L/LERK-3 and ELK-L/LERK-2/Cek5-L) have been found to require membrane anchorage or clustering to show activity (12). In view of the developmental expression patterns of ELF-2 and its receptors, discussed further below, the membrane anchorage of ELF-2 could play a role in ensuring the precise spatial specificity of developmental processes such as the formation of rhombomeres and somites, the guidance of cell migrations, or axon guidance and target recognition.

The close homology of ELF-2 and ELK-L/LERK-2/Cek5-L is evident not only in the extracellular receptor-binding domain but also in the intracellular domain. One feature that both ELF-2 and ELK-L/LERK-2/Cek5-L share with several other transmembrane ligands is a valine residue at the carboxy terminus. A C-terminal valine was shown to be required for the proteolytic cleavage of TGF-α in its extracellular domain (6), though it is not required for the equivalent cleavage of KL (10) and therefore might have some other function. Overall, the intracellular domains of ELF-2 and ELK-L/LERK-2/Cek5-L show a strikingly high degree of homology, including a remarkable stretch of complete identity over the last 33 amino acids. Other transmembrane ligands have not shown obvious intracellular homology of this type either with one another or with ELF-2.

This very high level of intracellular conservation between ELF-2 and ELK-L/LERK-2/Cek5-L suggests a significant functional role for the cytoplasmic domains of both proteins. One possible function could be a role in regulating the receptor-ligand interaction. In view of the activating effects of clustering ELK-L/LERK-2/Cek5-L and EHK1-L/LERK-3 (12), it is possible that the ligand intracellular domains of ELF-2 and ELK-L/LERK-2/Cek5-L could function to regulate ligand oligomerization. An alternative function for the intracellular domains might be associations with other proteins that could, for example, mediate cytoskeletal attachment or the transmission of a signal into the interior of the ligand-presenting cell.

When ELF-2 was tested for binding to Eph family receptors, strong interactions were detected with three of them: Elk (from mice), Cek10 (from chickens; apparent orthologs are mouse Sek-4 and human Hek2), and Cek5 (from chickens; the apparent mouse ortholog is Nuk/Sek-3). These three receptors are also closest neighbors to one another on family trees of the known Eph receptors and have presumably retained at least some aspects of their ligand-binding specificity following recent evolutionary duplications. ELK-L/LERK-2/Cek5-L also appears to bind to the same three receptors (5, 7, 12). When the affinities of Elk, Cek10, and Cek5 for ELF-2 were tested, all showed K_d values of approximately 1 nM. This value is within the typical range of affinities of ligands binding to their cognate tyrosine kinase receptors and moreover is at the high end of the range of affinities so far reported for ligands binding to Eph family receptors. These binding studies are therefore consistent with the idea that ELF-2 could be a genuine, biologically significant ligand for all three receptors. Especially in view of the complex interactions in the Eph family, with individual ligands binding more than one receptor and individual receptors binding more than one ligand, it is worth bearing in mind that ligand binding by cell surface receptors can result in either activating or antagonistic effects on signaling (for example, see reference 13). We found in this study that ELF-2 can induce signaling through a chimeric Elk receptor, which shows

that in this context ELF-2 is capable of activating intracellular signal transduction.

Studies of expression patterns provide further information on likely ligand-receptor interactions and can also indicate potential functions of the ligands and receptors in development. The expression of *ELF-2* RNA between days 8.5 and 10.5 of mouse development is consistent with roles in early organogenesis. The distribution of *ELF-2* expression at this stage in a rhombomere-specific pattern in the hindbrain, and more weakly in the midbrain and forebrain, suggests roles for ELF-2 in the development of the nervous system. Several of the Eph family kinases also display rhombomere-specific expression in the hindbrain, including Nuk/Sek-3/Cek5 and Sek-4/Cek10 (4, 20), which we have demonstrated here to be receptors that bind ELF-2. The expression pattern of the Elk receptor, which we also find to bind ELF-2, during development has not been examined in detail. However, Elk was reported to be expressed specifically in the brain in adult rats, implying that this receptor too may have a role in nervous system development or function (25).

The rhombomere-specific expression of *ELF-2* RNA in the hindbrain region suggests potential functions either in the establishment of the segmental pattern or in the subsequent development of segment-specific properties such as neural crest emigration or the establishment of segment-specific neuronal connections. In this regard, it is intriguing that *ELF-2* expression in the hindbrain is most prominent at the level of rhombomeres 1, 2, 4, and 6. The same rhombomeres mark the major levels at which streams of neural crest cells migrate from the hindbrain to the branchial arches; additionally, rhombomeres 2, 4, and 6 mark points where cranial nerves connect with the hindbrain (26, 27). ELF-2 expression in the hindbrain, as well as in the branchial arches, could therefore be involved in the spatial patterning of these developmental systems. A role in patterning neuronal connections is particularly plausible in view of the expression of Nuk/Sek-3/Cek5, as well as other Eph family receptors, on some of the earliest axonal projections formed in the embryo (10b, 20, 35).

A role for ELF-2 in mesoderm segmentation is implied by its expression in bands at the site of somitogenesis. Two prominent bands of *ELF-2* RNA expression are apparent near the boundary between somites and the presomitic mesoderm and move down the length of the embryo as the boundary progresses. Weaker expression is also detected in more anterior somites in the segmented mesoderm. Similar RNA expression patterns have been described for the Eph family receptor Sek (30), as well as for the receptor Sek-4/Cek10 (4), shown here to bind ELF-2. *Sek-4* is expressed in a cluster of newly formed somites, with the most recently formed one displaying the highest level of expression (4). The expression of ELF-2 in the segmented mesoderm, as in the rhombomeres, is therefore consistent with interactions with Eph family receptors; Sek-4/Cek10 in particular is a possible receptor for ELF-2 in the somites. Potential roles for ELF-2 in the somites could include the induction or condensation of each new somite or subsequent formation of compartments within somites.

In view of the importance of the known ligands for receptor tyrosine kinases, it is remarkable that by far the largest known family of receptor tyrosine kinases, the Eph family, should until recently have had no known ligands. The expression patterns of the receptors have nonetheless been intriguing, with almost all of them being expressed prominently in the nervous system and many of them being expressed in distinctive and highly restricted patterns in the embryo at the start of organogenesis. The identification of ELF-2 and other members of the Eph ligand family has provided further evidence of potential roles in early organogenesis and neuronal development and will now allow direct investigations of the biological functions of this new class of ligands.

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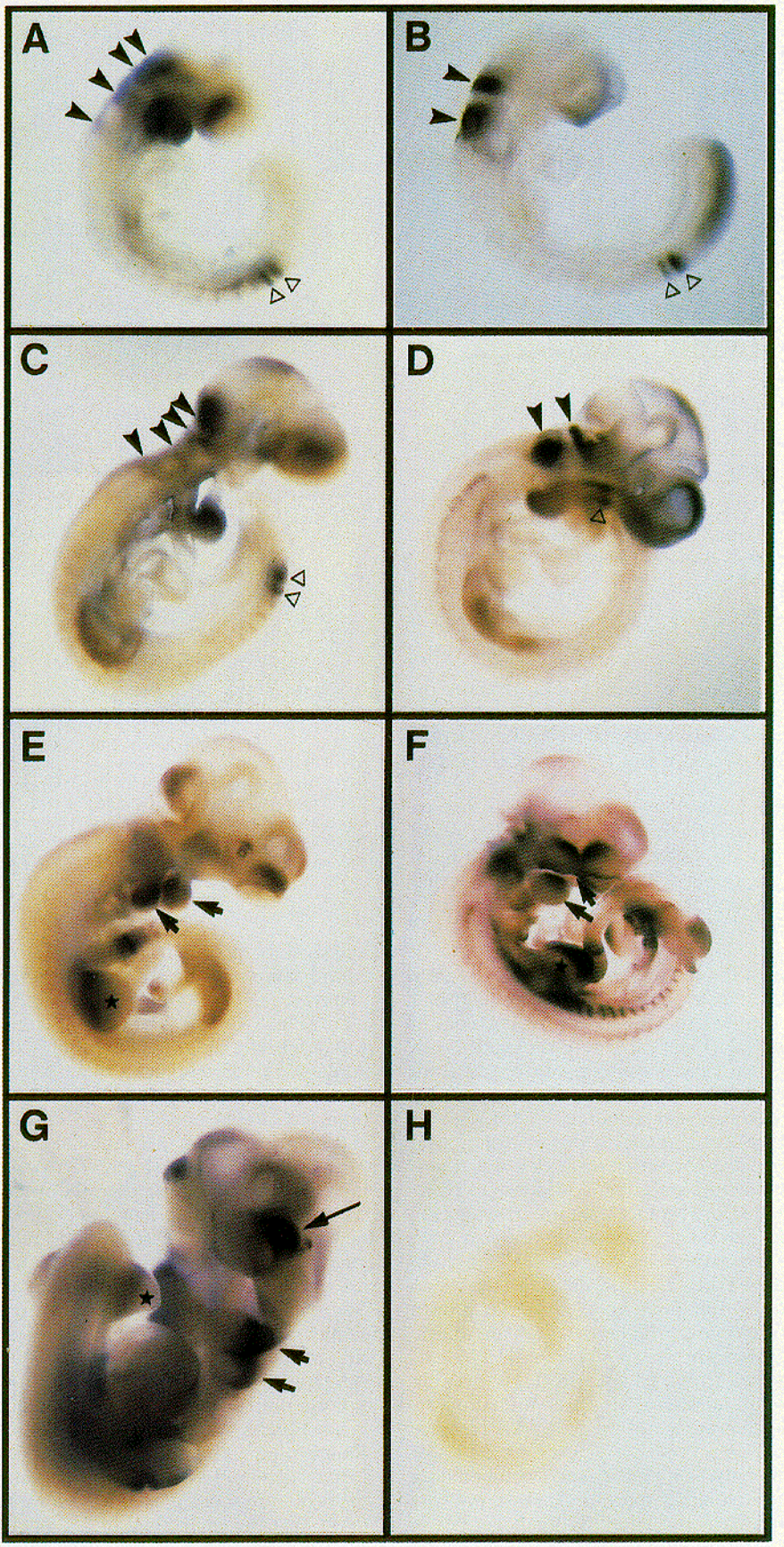
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