

Complementary Gradients in Expression and Binding of ELF-1 and Mek4 in Development of the Topographic Retinotectal Projection Map

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Summary

Topographic maps with a defined spatial ordering of neuronal connections are a key feature of brain organization. Such maps are believed to develop in response to complementary position-specific labels in presynaptic and postsynaptic fields. However, the complementary labeling molecules are not known. In the well-studied visual map of retinal axons projecting to the tectum, the labels are hypothesized to be in gradients, without needing large numbers of cell-specific molecules. We recently cloned ELF-1 as a ligand for Eph family receptors. Here, RNA hybridization shows matching expression gradients for ELF-1 in the tectum and its receptor Mek4 in the retina. Binding activity detected with alkaline phosphatase fusions of ELF-1 and Mek4 also reveals gradients and provides direct evidence for molecular complementarity of gradients in reciprocal fields. ELF-1 and Mek4 may therefore play roles in retinotectal development and have properties predicted of topographic mapping labels.

Introduction

The idea that the brain contains spatially accurate representations of the outside world dates at least to the seventeenth century, when Descartes (1664) represented the visual world as projecting from the eye onto a spatially accurate map in the brain. Such topographic maps are now known to develop when one set of neurons sends out axonal connections to another set, so that the order of the neurons is reflected in the spatial order of the connections. It is also now known that such topographic maps are found throughout the nervous system. These maps can form representations of varied types of sensory input, as well as motor output, and can also transfer information among regions within the brain, allowing successive functional operations to be performed while maintaining the information in its original spatial order (Udin and Fawcett, 1988; Hunt and Cowan, 1990; Jacobson, 1991; Lewin, 1994).

The development of topographic maps in the embryo can, in principle, be explained by stable identification tags on the presynaptic neurons interacting with complementary tags on the postsynaptic neurons, as first proposed more than fifty years ago by Sperry (1943, 1963) in the chemoaffinity theory. These identification tags would constitute position-specific information, which would then be translated into a map by mechanisms affecting axon growth and connectivity. The chemoaffinity theory was de-

veloped mainly on the basis of studies on the retinotectal system, a topographic map formed by projection of axons from the retinal ganglion cells onto the tectum of the mid-brain to produce an accurate image of the visual world. In the decades since the chemoaffinity theory was proposed, the retinotectal system has been a favorite model for the study of topographic map development. The basic idea of mapping by complementary labels on the retina and tectum has been amply confirmed by numerous studies, including a variety of tissue-grafting and ablation experiments, as well as studies of cellular properties in vitro such as axon guidance and axon branching (Sperry, 1963; Walter et al., 1987; Boxberg et al., 1993; Roskies and O'Leary, 1994; reviewed by Hunt and Cowan, 1990; Jacobson, 1991; Mey and Thanos, 1992; Goodman and Shatz, 1993; Holt and Harris, 1993).

The position-specific tags that guide topographic mapping could theoretically take the form of cell type-specific labels or could be molecular gradients. Sperry, noting that there are probably not nearly enough "bits of information" in the genome for cell-specific tags throughout the nervous system, proposed that position-specific information in the retinotectal system is likely to take the form of gradients (Sperry, 1943, 1963). With gradients along two or more axes of the retina and gradients along matching axes of the tectum, each location on the retina and tectum could have a unique position-specific value. Such gradients would be formally analogous to gradients that specify position in other embryonic fields, with the additional requirement that they would have to be complementary in two separate fields, so that each position on the retina could map to the corresponding position on the tectum. The concept that the complementary retinotectal labels may be in gradients is widely accepted, although direct evidence for such gradients is lacking.

Several molecules have been identified with expression patterns that correlate with position in topographic maps. For example, homeobox genes such as LIM genes in motor neurons (Ericson et al., 1992; Tsuchida et al., 1994), *engrailed* in the tectum (Itasaki and Nakamura, 1992), and *SOHo-1* in the retina (Deitcher et al., 1994) all have expression that correlates with map position and have been proposed to encode nuclear factors that could specify topographic properties. Other molecules with asymmetric distributions in the retinotectal system include TOP antigen (Savitt et al., 1995), TRAP antigen (McLoon, 1991), and a partially purified 33 kDa tectal protein (Stahl et al., 1990). However, the identification of complementary cell-cell interaction molecules that could act as labels of the type predicted by the chemoaffinity theory has remained an elusive goal (Mey and Thanos, 1992; Goodman and Shatz, 1993; Holt and Harris, 1993).

Eph ligand family 1 (ELF-1) was recently cloned as a ligand that binds with high affinity to the Mek4 receptor and lower affinity to the Sek receptor (Cheng and Flanagan, 1994). These two receptors are members of the Eph fam-

ily, by far the largest known family of receptor tyrosine kinases, with at least 12 members so far identified in vertebrates. The Eph receptors have been notable for their distinctive expression domains at the time of early organogenesis and because almost all of them are expressed prominently or exclusively in the nervous system (for example, Lai and Lemke, 1991; Nieto et al., 1992; Sajjadi and Pasquale, 1993; Cheng and Flanagan, 1994; Henkemeyer et al., 1994; Soans et al., 1994). However, the understanding of their function has been limited by the remarkable fact that all of them were identified as orphan receptors without known ligands. Only recently have there been reports of ligands binding to these receptors, forming a corresponding Eph ligand family (Bartley et al., 1994; Beckmann et al., 1994; Cheng and Flanagan, 1994; Davis et al., 1994; Bennett et al., 1995; Bergemann et al., 1995; Kozlosky et al., 1995).

We describe here a characterization of ELF-1, Mek4, and Sek in the chick retinotectal system. In situ RNA hybridization indicates the ligand is expressed in the tectum and the receptors in the projecting retinal ganglion cells. Both ELF-1 and Mek4 show gradients in RNA expression, and the gradients are along matching axes that map to one another. To investigate ligand-receptor interactions, we used alkaline phosphatase (AP) fusion proteins of ELF-1 and Mek4 to probe embryonic tissues. This approach not only allows us to test directly for gradients of binding activity within each field, but also allows us to address directly the critical question of the complementarity of those gradients in the reciprocal field. The results support the existence of gradients of active ELF-1 and Mek4 within the field where each is expressed and also provide direct evidence for molecular complementarity of those gradients in the reciprocal field. These results suggest a role for ELF-1 and Mek4 in retinotectal development, possibly in combination with other Eph receptors or ligands, and indicate that they have properties consistent with complementary gradient molecules of the type predicted by the chemoaffinity theory of Sperry.

Results

ELF-1 RNA Expression in the Tectum

In the mouse embryo, we found previously that ELF-1 is expressed from the neural fold stage at the start of organogenesis, with highest levels in the midbrain (Cheng and Flanagan, 1994). Since the tectum (or superior colliculus in mammals) forms a major part of the midbrain, this observation suggested a possible involvement of ELF-1 in the retinotectal projection. To examine this further, we cloned a homolog of ELF-1 from the chicken, a species in which the developing retina and tectum are especially prominent and in which the retinotectal system is the primary visual projection and has been characterized extensively.

Chick cDNA clones were isolated from a library by hybridization with a mouse *ELF-1* cDNA probe. Alignment of the mouse and chick sequences (Figure 1) indicates an amino acid identity of 79%, excluding the predicted secretion signal sequences. The identity rises to 90% over a core region (amino acids 47–161 of chick ELF-1) that

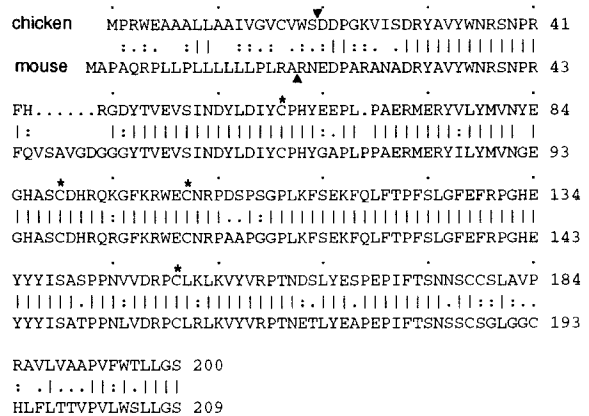


Figure 1. Identification of a Chick Homolog of Mouse ELF-1

The amino acid sequence deduced from mouse *ELF-1* cDNA (Cheng and Flanagan, 1994) is aligned with the amino acid sequence from chick *ELF-1* cDNA. Sequences were aligned with the GAP program. Closed triangles indicate predicted ends for secretion signal sequences, asterisks mark cysteines conserved in other members of the Eph ligand family, identical amino acids are shown by a line, and conservative changes are indicated by dots between the two sequences.

includes the four cysteines so far found in all Eph family ligands.

The distribution of *ELF-1* RNA in chick embryos was examined by whole-mount in situ hybridization. The RNA expression pattern at early organogenesis is similar to that in the mouse (Cheng and Flanagan, 1994), with very high levels seen in the midbrain (Figure 2). Other areas that show lower intensity include the anterior hindbrain, branchial arches, and limb buds (Figure 2A). In the chick, the midbrain expression can be seen to localize to the tectum (Figures 2A–2D). Tectal expression of *ELF-1* RNA is detected weakly on day 2 of development (data not shown) and is prominent by day 3 (Figure 2A), well before the retinal axons start to arrive in the tectum on day 6 (Lavail and Cowan, 1971). High expression continues to be seen on days 4, 6, and 8 (Figures 2B–2D).

Within the tectum, *ELF-1* RNA expression is not uniform. By day 3, and more obviously on days 4, 6, and 8, *ELF-1* RNA is expressed in a gradient, with low levels at the anterior end of the tectum and high levels at the posterior end (Figures 2A–2D and 2F). Reexamination of our data from the mouse indicates that, as in the chick, *ELF-1* RNA expression is highest in the posterior tectum and low in the anterior tectum (Cheng and Flanagan, 1994; unpublished data).

Mek4 and Sek RNA Expression in the Ganglion Cell Layer of the Retina

We were next interested to test whether Mek4 and Sek, receptors known to bind ELF-1, might be expressed in the developing retina, particularly in the ganglion cells, the cell type that sends out projections to the tectum. Consistent with this possibility, in an earlier Northern blot survey of chicken adult tissues, *Mek4* and *Sek* (also called *Cek4* and *Cek8*, respectively, in the chicken) were found at highest levels in the adult brain and adult retina (Sajjadi and

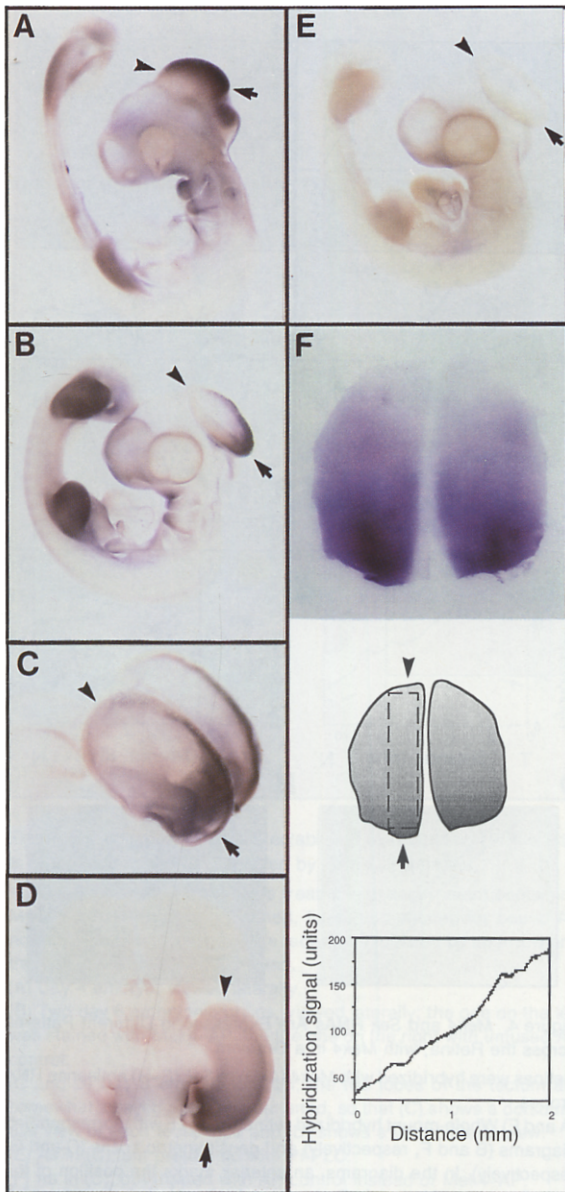


Figure 2. *ELF-1* RNA Is Expressed in the Developing Tectum and Is in a Gradient

Whole mounts were hybridized with *ELF-1* antisense probe or, in (E), control sense strand. The posterior end of the tectum is indicated by an arrow and the anterior end by an arrowhead.

(A) Day 3 embryo, tectum viewed laterally.

(B) Day 4 embryo, tectum viewed laterally.

(C) Day 6 embryonic brain, viewed dorsolaterally; the tectum is delicate at this stage, and wrinkles cause the impression of discontinuities in staining.

(D) Day 8 embryonic brain, with the two lobes of the tectum somewhat splayed apart and flattened, resulting in a ventrolateral view. The darker stripe around the edge of the tectum in parts (A)–(D) is caused by the greater thickness of tissue that results from viewing a hollow curved object.

(E) Day 4 embryo, control sense probe.

(F) The same tectum as in (B), excised and flattened, with a corresponding diagram and densitometric scan. The scan is taken along the dashed box, corresponding approximately to the anteroposterior axis.

Pasquale, 1993). To examine expression in the developing retina, sections were hybridized with RNA probes for chick *Mek4* and *Sek*. The results show conspicuous expression of both *Mek4* and *Sek* (Figures 3A–3D). The early developmental appearance of the stained cells, their large nuclei, and their location next to the fiber layer at the inner retinal surface indicates expression in the retinal ganglion cells. Although displaced amacrine cells make up 30%–35% of cells in the ganglion cell layer of the adult chicken, they do not migrate to that location until after day 10 (Spira et al., 1987). For further confirmation, we used an antibody to Islet-1 (Ericson et al., 1992; Tsuchida et al., 1994), a marker that identifies ganglion cells at this stage of development in the chick retina (C. Austin and C. Cepko, personal communication). Nuclear staining for Islet-1 appears in the same layer of the retina as the *Mek4* and *Sek* hybridization (Figures 3E and 3F) and colocalizes in double-labeling experiments (Figures 3G and 3H), providing further evidence for expression of *Mek4* and *Sek* in ganglion cells. These results do not exclude the possibility of *Mek4* or *Sek* expression at some stage of development in other retinal cell types, nor the possibility that other Eph family receptors could be expressed in the ganglion cells, especially since these receptors form a large family that may have additional uncharacterized members.

To test for gradients across the retina, we hybridized whole-mount preparations of developing chick retinas to *Mek4* or *Sek* RNA probes. In multiple experiments, *Mek4* was consistently seen in a gradient along the nasotemporal axis, with highest levels near the temporal pole (Figures 4A–4C). *Sek* hybridization was also seen, but showed no obvious gradation in repeated experiments (Figures 4E–4G). In retinal cross sections as well, where the ganglion cell layer can be seen specifically, *Sek* expression was seen at comparable levels on the nasal and temporal sides (Figure 4H), whereas *Mek4* expression was higher on the temporal side (Figure 4D).

RAP In Situ with a *Mek4*-AP Probe Detects Ligand Activity in the Tectum That Is in a Gradient and Is Sensitive to PI-PLC

The RNA hybridization results suggested that receptors for ELF-1 might be on axons projecting from the retinal ganglion cells and might be capable of detecting a gradient of ELF-1 in the tectum. An alternative, or additional, possibility might be local interactions within the retina or the tectum. However, we have so far seen no indication of this, as there was no obvious expression of *ELF-1* RNA in the retina nor of *Mek4* or *Sek* RNA in the tectum (Cheng and Flanagan, 1994; data not shown). To test more directly for ligand activity in the chick tectum detectable by *Mek4* and *Sek*, we used a technique we described recently, RAP in situ (for receptor affinity probe or receptor alkaline phosphatase in situ), in which soluble receptor-AP fusion proteins are used to detect the distribution of ligand activity directly in tissues (Cheng and Flanagan, 1994).

The results show that *Mek4*-AP can indeed detect high ligand activity in the tectum and that the activity is in a gradient, with high levels at the posterior end and low levels at the anterior end (Figures 5A–5D, 5G, and 5H).

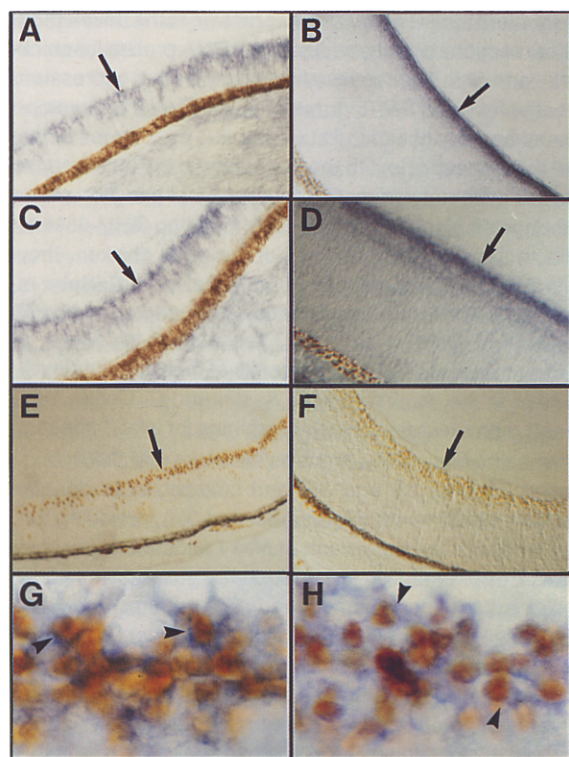


Figure 3. *Mek4* and *Sek* RNAs in the Ganglion Cell Layer of the Developing Retina

Sections of retinas at day 6 or 8 were treated with RNA probes for *Mek4* (A and B) or *Sek* (C and D) or antibody to the ganglion cell marker Islet-1 (E and F). Arrows mark the ganglion cell layer; RNA hybridization signal is in blue, and Islet-1 nuclear staining is in brown, while the pigment epithelium at the back of the retina is visible as a brown stripe. Parts (A)–(F) are viewed with differential interference contrast (DIC) optics. (G and H) Double labeling with Islet-1 antibody, together with *Sek* or *Mek4* RNA probes, respectively. The ganglion cell layer runs across the middle, with the optic fiber layer above and the inner plexiform layer below. Brown nuclear Islet-1 staining is seen overlapping or adjacent to blue cytoplasmic staining for *Mek4* or *Sek* RNAs (examples indicated with arrowheads).

The gradient is readily detectable throughout the period from day 4 to at least day 15 (Figures 5A–5D, 5G, and 5H; data not shown), with the highest intensity seen from approximately day 6 to day 11. Similar results were obtained by RAP in situ with the *Sek* receptor as probe (data not shown).

Mouse ELF-1 is attached to cell membranes by a glycosylphosphatidylinositol (GPI) anchor (Cheng and Flanagan, 1994), a feature shared by some other members of the Eph ligand family (Bartley et al., 1994; Davis et al., 1994; Kozlosky et al., 1995), but so far not seen in other ligands that bind to tyrosine kinase receptors. To determine whether the activity detected by RAP in situ in the chick tectum may also be localized by a GPI anchor, embryos were treated with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves GPI anchors. PI-PLC pretreatment reduced the tectal RAP in situ signal essentially to background levels (Figure 5E compared with Figures 5C and 5F), indicating that the ligand

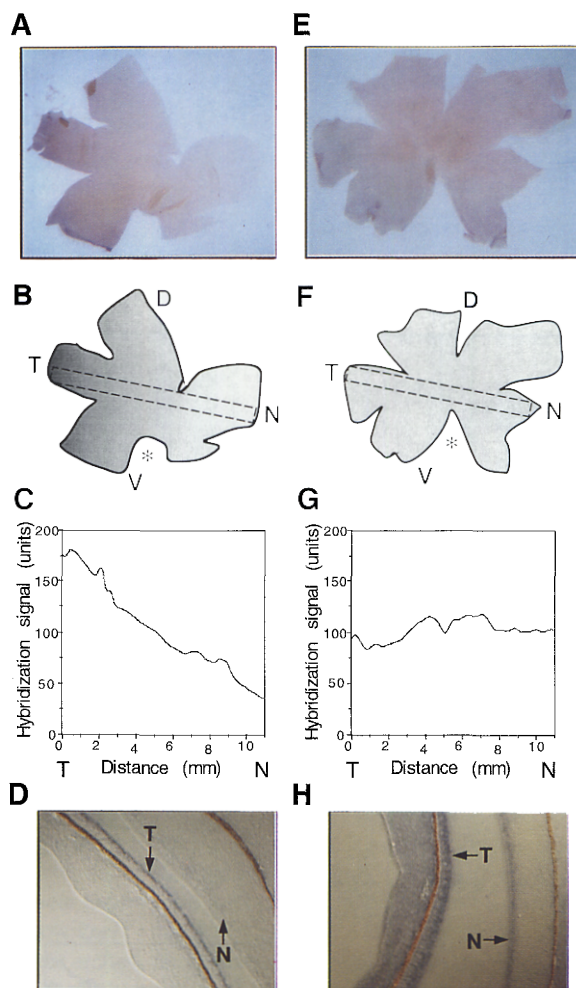


Figure 4. *Mek4* and *Sek* RNAs Are Expressed in Different Patterns across the Retina, with *Mek4* in a Gradient

Retinas were hybridized with *Mek4* (A–D) or *Sek* (E–H) antisense RNA probes.

(A and E) Whole-mount hybridized retinas at day 8 with corresponding diagrams (B and F, respectively) and densitometric scans (C and G, respectively). In the diagrams, an asterisk marks the position of the optic fissure, and letters indicate nasal, temporal, dorsal, and ventral poles. The densitometric scans were taken along the dashed boxes, corresponding approximately to the nasotemporal axis. *Mek4* expression was in a gradient, whereas *Sek* expression showed no obvious gradation.

(D and H) Coronal sections through an embryo, each showing nasal (N) and temporal (T) portions of a single retina. *Sek* hybridization is visible in the ganglion cell layer in both nasal and temporal parts, while *Mek4* hybridization is visible at higher levels in the temporal than in the nasal portion. In each case shown here, the nasal portion is more peripheral than the temporal portion that lies adjacent to it and is therefore thinner and somewhat less advanced developmentally, but this central–peripheral difference does not correlate with staining intensity.

detected by RAP in situ in the chick tectum is anchored by a GPI tail.

When chick retinal axons arrive in the tectum, they grow from the anterior end toward the posterior end and initially grow across the outer tectal surface within a layer known as the stratum opticum. Later, after reaching the topo-

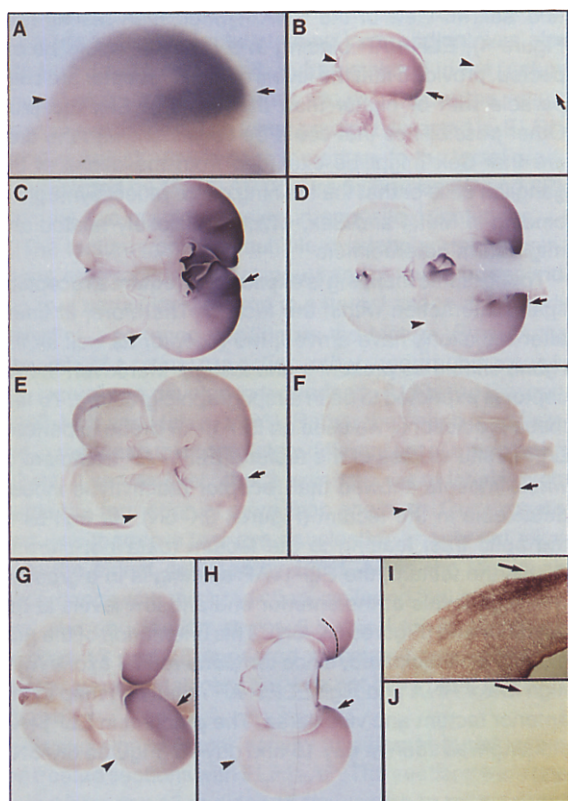


Figure 5. Ligand Activity Is Detectable in the Tectum by RAP In Situ, Is in a Gradient, and Is Removed by PI-PLC Treatment

Whole mounts or sections were treated with supernatant containing Mek4-AP and then were washed, fixed, and stained for bound AP activity. The posterior end of the tectum is indicated by an arrow and the anterior end by an arrowhead.

(A) Day 4 embryo, viewed laterally.

(B) Two day 6 embryonic brains, viewed laterally; the one on the left was stained with Mek4-AP and the one on the right with unfused AP control.

(C and D) Day 8 embryonic brain; the two lobes of the tectum are somewhat splayed apart and flattened, so that (C) shows a dorsomedial view of each tectal lobe and (D) shows a ventrolateral view.

(E) As in (C), but pretreated with PI-PLC.

(F) As in (C), but treated with AP control instead of Mek4-AP.

(G) Day 11 embryonic brain, dorsal view.

(H) Day 13 embryonic tectum, caudoventral view

(I and J) Adjacent sagittal sections from the posterior part of a day 13 tectum; the approximate position is indicated by a broken line over a different tectum shown in (H). (I) shows Mek4-AP staining, and (J) shows unfused AP control. An arrow indicates the outer tectal surface near the posterior end.

graphically correct position, the axons or their side branches can penetrate radially into the tectum (Nakamura and O'Leary, 1989). It is therefore likely that a topographic guidance molecule in the tectum would be located at outer layers where the axons arrive and might also be in deeper layers. We investigated this by modifying the RAP in situ procedure: instead of treating whole tissues with AP fusion protein, frozen sections of tectum were prepared and then treated with Mek4-AP or with AP as a control (Figures 5I and 5J). Consistent with the tectal whole mounts, the sections show a posterior to anterior gradation of reactivity. On the sections, reactivity with Mek4-AP is seen in all layers of the tectum, with strong reactivity in outer layers where the retinal axons arrive and penetrate.

LAP In Situ with an ELF1-AP Probe Detects Receptor Activity on Retinal Axons and in a Gradient in the Stratum Opticum of the Tectum

We next tested whether the RAP in situ technique, which we originally described using receptor-AP fusion probes to detect ligand activity, could be adapted to perform a technique, LAP in situ (for ligand affinity probe or ligand alkaline phosphatase in situ), in which a ligand-AP fusion would be used to detect receptors. For this purpose, an *ELF-1* cDNA was inserted in a new vector, *APtag-2*, to produce a fusion construct encoding all of ELF-1 except the C-terminal GPI anchor addition signal, fused to the N-terminus of a dimeric AP tag.

The ELF1-AP fusion protein retains receptor-binding activity, as indicated by binding to cells expressing either Mek4 or Sek (Table 1). ELF1-AP also binds to a Mek4-immunoglobulin fusion protein in a cell-free system, further confirming a direct ligand-receptor interaction (Table 1). These results are consistent with our previous demonstration that Mek4-AP or Sek-AP fusions bind to cells transfected with native ELF-1 (Cheng and Flanagan, 1994). In addition, when ELF1-AP was tested by LAP in situ on whole mouse or chick embryos at early organogenesis (data not shown), all of the obvious stained areas corresponded to regions where either *Mek4* or *Sek* RNA is expressed (Nieto et al., 1992; Cheng and Flanagan, 1994; unpublished data), consistent with the idea of Mek4 and Sek being major receptors for ELF-1 in development, although not excluding the possibility of other receptors. Correspondingly, almost all of the prominent areas of RAP

Table 1. Binding of ELF1-AP to Mek4 and Sek Receptors

AP Reagent	Binding to COS plus Mek4 (OD/hr)	Binding to COS plus Sek (OD/hr)	Binding to Mek4-Immunoglobulin Beads (OD/hr)	Binding to Control Beads (OD/hr)
ELF1-AP	4.97 ± 0.27	52.3 ± 1.4	55.4 ± 4.6	0.00
AP	0.08 ± 0.03	0.10 ± 0.01	0.00	0.00

Supernatants containing ELF1-AP fusion protein or unfused AP as a control, each at 1000 OD/hr/ml or approximately 30 nM, were incubated with COS cells or protein A-agarose beads, which were then washed and assayed for bound AP activity. Each binding assay was performed in triplicate, and results are shown as the mean ± SD. Results are shown for COS cells transfected with Mek4 or Sek receptors, after subtracting the background binding for untransfected COS cells. The protein A-agarose beads were preincubated with supernatant from COS cells transfected with a Mek4-immunoglobulin fusion construct or with control supernatant from untransfected COS cells.

in situ staining seen in mouse or chick embryos with *Mek4*-AP or *Sek*-AP probes coincide with the sites of expression of *ELF-1* RNA, implying that *ELF-1* is a major ligand for *Mek4* and *Sek*, although there may also be additional ligands (Cheng and Flanagan, 1994).

To examine the distribution of receptors that can bind *ELF-1* in the retinotectal system, we first tested retinal explants grown in culture, where individual axons can be seen clearly and are known to show topographically appropriate responses to tectal membranes (Walter et al., 1987; Boxberg et al., 1993). If *ELF-1* is involved in retinotectal mapping, it would be expected that *ELF-1*-AP would detect receptor activity on retinal axons and that the binding might show temporal-nasal differences. The results show that *ELF-1*-AP does bind to retinal axons (Figures 6A-6J). Staining well above control levels was seen with both nasal and temporal axons (Figures 6C and 6D compared with 6E). However, in multiple experiments, temporal axons consistently stained more strongly than nasal axons, with the nasal axons requiring approximately 3- to 5-fold longer incubations with AP substrate to reach a similar intensity (Figures 6A and 6C compared with 6B and 6D). This temporal-nasal difference, as well as the tectal LAP in situ gradients described below, appears consistent with all of our other observations. The most obvious interpretation would be simple additive binding of *ELF-1*-AP to both *Mek4*

and *Sek*: in view of the RNA hybridization results (see Figure 4), *ELF-1*-AP binding in a gradient would be expected, provided that the levels of *Mek4* receptor are comparable with or higher than the levels of *Sek* receptor. Other possibilities that could be consistent with the data are that *Sek* might be excluded from the axons of the ganglion cells or that the staining might reflect synergistic binding to *Mek4* and *Sek*, which are closely related and might form heterodimers.

Topographic mapping is expected to require a receptor-ligand interaction within the tectum. Therefore, at times after the axons have arrived, the receptor as well as the ligand should be present in the tectum. Moreover, the receptor is expected to be in a topographic gradient. To test these predictions, we used an *ELF-1*-AP probe to perform LAP in situ studies of the tectum. *ELF-1*-AP treatment of whole mounts showed that receptor reactivity is indeed detectable in the tectum (Figures 6K-6N), as well as in the optic tract leading to the tectum (data not shown). Within the tectum, the *ELF-1*-AP staining is in a gradient with high levels at the anterior end and low levels at the posterior end (Figures 6K-6N). This orientation of the gradient is as anticipated, since temporal retinal axons (with high *Mek4* RNA and high *ELF-1*-AP reactivity) map to the anterior tectum and vice versa. The gradient in *ELF-1*-AP staining was seen at day 13 and day 15 (Figures 6K-6N),

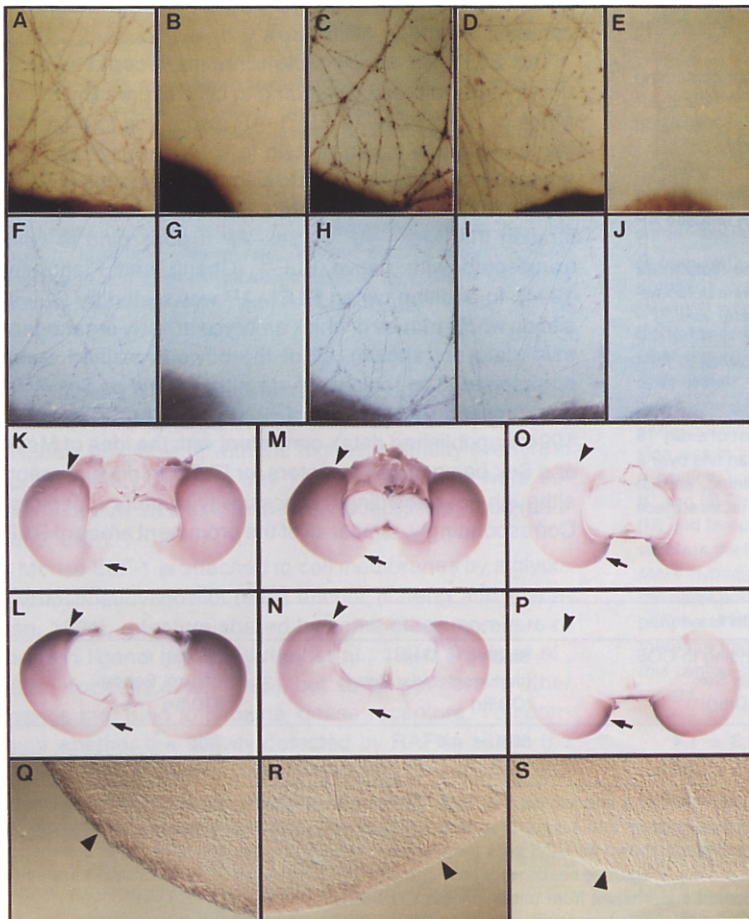


Figure 6. LAP In Situ with an *ELF-1*-AP Probe Detects Receptors on Retinal Axons In Vitro and in a Gradient in the Stratum Opticum of the Tectum

(A-E) Axons grown from retinal explants in vitro. The edge of the original explant is at the lower border, and outgrowing axons cross the rest of the field. (A) and (C) are temporal explants; (B) and (D) are nasal. (A)-(D) are treated with *ELF-1*-AP; (E) is a temporal explant treated with unfused AP control. (A) and (B) are stained with AP substrate for 6 hr; (C), (D), and (E) for 19 hr.

(F-J) Identical to (A)-(E), but viewed with DIC optics to show unstained axons.

(K-N) Whole-mount LAP in situ of chick tectum with *ELF-1*-AP probe. (K) and (L) are from day 15 embryos; (M) and (N), day 13. (K) and (M) are caudoventral views; (L) and (N) are rostro-dorsal views. The posterior end of the tectum is indicated by an arrow and the anterior end by an arrowhead.

(O and P) Whole-mount RAP in situ with a *Mek4*-AP probe, shown for comparison with the *ELF-1*-AP treatment of tectums at the same stage shown in (M) and (N), respectively.

(Q-S) Sagittal sections of tectum at day 13, tested by LAP in situ with an *ELF-1*-AP probe and viewed with DIC optics. The three sections are from anterior, intermediate, and posterior regions, respectively. Arrowheads indicate the position of the stratum opticum at the outer tectal surface (layer XII of the day 12-14 chick embryo, according to Lavail and Cowan [1971]). Staining of the stratum opticum is seen in the anterior section in (Q), is seen more weakly in the intermediate section in (R), and is not obviously visible in the posterior section in (S).

as the topographic order of the map is reaching completion (Jacobson, 1991). Tectal staining in a gradient was also seen on day 11 and day 8, but no staining in the tectum was seen on day 4 (data not shown), before the retinal axons have arrived. The tectal gradient detected by ELF1-AP (Figures 6K-6N) is complementary to the tectal gradient detected by Mek4-AP (Figure 5; Figures 6O and 6P), with the two gradients being in opposite orientations.

The tectum contains multiple overlapping topographic maps, all in register so that inputs and outputs corresponding to a particular direction in external space are coordinated at a common position on the tectum. Even in the absence of a retinal projection, other topographic maps in the tectum can develop independently (Udin and Fawcett, 1988; Holt and Harris, 1993). Rather than each map forming in response to a completely different set of topographic labels, it seems likely that a common set of labels might guide the formation of more than one map. Our expectation was therefore that eye enucleation should not eliminate the signal detected by ELF1-AP in the tectum. In accordance with this prediction, we found that after unilateral eye enucleation at day 3.5, the LAP in situ signal at days 13 and 15 appeared somewhat reduced in the contralateral tectal lobe, but was not eliminated (data not shown).

To examine receptor activity in different tectal layers, we treated sections with ELF1-AP. The sections were prepared from day 13 and day 15 tectum, when retinal axons have reached the posterior tectum, but before they have become myelinated (Mey and Thanos, 1992). Since the retinal axons grow into the tectum through the stratum opticum, LAP in situ is expected to show receptor reactivity in this outer layer. Consistent with this, the results show that tectal staining with ELF1-AP localizes to the stratum opticum (Figures 6Q-6S). The intensity of ELF1-AP staining in the stratum opticum showed a posterior to anterior gradation in the sections (Figures 6Q-6S), as seen on the whole mounts (Figures 6K-6N), indicating that the gradients on the whole mounts are not simply due to differences in thickness of the stratum opticum, but reflect differences in receptor density detected by ELF1-AP along the antero-posterior axis.

Discussion

Most axons in the central nervous system form precise topographic connections, and the development of topographic maps is a key feature of the way in which the nervous system is assembled. However, little is known about the molecular mechanisms that control the establishment of topographic order during embryonic development. While additional characterization of ELF-1 will be required to establish firmly whether it is a topographic guidance molecule, we show here that the properties of ELF-1 and its receptors seem consistent with a role in development of the retinotectal projection, probably the best-studied map with regard to the establishment of topographic order during development.

One line of evidence comes from RNA expression patterns in the chick retinotectal system. In situ hybridization

of *ELF-1* RNA shows strong expression in the target field (the tectum), with levels higher than any other region of the embryo. The same is true of *ELF-1* RNA in the equivalent region of the mouse (Cheng and Flanagan, 1994). RNAs for the *Mek4* and *Sek* receptors are in the projecting field (the retina). Within the retina, *Mek4* and *Sek* RNAs localize to the projecting cell type, the retinal ganglion cell. Also, *ELF-1*, *Mek4*, and *Sek* are all expressed at the correct time of development to be involved in retinotectal mapping. Finally, consistent with the chemoaffinity theory, *ELF-1* and *Mek4* RNAs are both expressed in gradients in their respective fields, and those gradients are along matching axes that map to one another (see Figure 7).

To analyze ligand-receptor interactions directly, we used receptor-AP or ligand-AP fusion proteins to detect the pattern of binding activity in embryos. We previously described this approach as RAP in situ, for receptor affinity probe or receptor alkaline phosphatase in situ, using receptor fusions as probes (Cheng and Flanagan, 1994). Here we show that a ligand can also be used as probe in a reciprocal technique, LAP in situ. Below we also use the term AP in situ as a more general description to include probes derived from receptors, ligands, or other types of interacting molecule. The information provided by AP in situ is qualitatively different from RNA in situ or immunolocalization, which can determine expression patterns for a ligand or receptor, but give no information on binding activity. In the context of the present study, AP in situ can be regarded as giving two types of information.

First, it can be used to infer distributions of the active forms of specific molecules. For example, in both mouse and chick embryos, the remarkably close similarity between the *ELF-1* RNA in situ pattern and the RAP in situ pattern with a *Mek4*-AP probe provides evidence on the distribution of active ELF-1 protein. This similarity is particularly striking in the tectum, where both techniques detect a signal that is very strong and higher than in any other

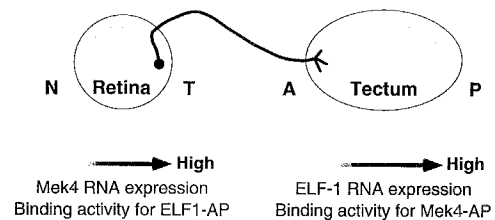


Figure 7. Complementary Expression and Binding of ELF-1 and Mek4 in Relation to Retinotectal Topographic Mapping

Topographic order is arranged so that axons from the nasal (N) retina project to the posterior (P) tectum, while axons from the temporal (T) retina project to the anterior (A) tectum. Topographic order is also established along the dorsoventral axis. Matching gradients are seen for *ELF-1* and *Mek4* RNAs in the tectum and retina, respectively. Ligand-receptor binding activity detected by AP fusion proteins also shows a complementary distribution, with *Mek4*-AP detecting ligand activity in a gradient in the tectum, while ELF1-AP detects receptor activity on retinal axons in vitro, with higher reactivity on temporal than on nasal axons, as well as in a gradient in the stratum opticum after the retinal axons arrive in the tectum. The high end of the retinal gradients (*Mek4* RNA and binding of ELF1-AP) maps to the low end of the tectal gradients (*ELF-1* RNA and binding of *Mek4*-AP) and vice versa.

region of the embryo. As with immunolocalization, it must be remembered that the pattern is expected to be a composite of all cross-reacting molecules and Mek4-AP may be binding to one or more other ligands in addition to ELF-1 (Cheng and Flanagan, 1994). Compared with immunolocalization, an advantage of AP *in situ* is that it is expected to be specific for detection of active forms of molecules capable of a ligand-receptor interaction.

The second and more direct type of information from AP *in situ*, which is not available from other techniques such as RNA hybridization *in situ* or immunolocalization, is on the potential sites of interaction of the molecule used as a probe. For example, regardless of the specific identity of the ligand(s), RAP *in situ* with a Mek4-AP probe shows a property of Mek4: that is, that it can detect ligand activity that is at very high levels in the tectum and is in a gradient. This type of information is particularly important in the study we describe here, because it allows us not only to demonstrate gradients, but also to test directly the molecular complementarity of those gradients in reciprocal fields. This complementarity is a central prediction of the chemoaffinity theory and a prediction that may not be directly addressed by other methods, such as RNA hybridization *in situ*, immunolocalization, or functional assays of axon behavior.

Figure 7 summarizes the distributions detected here for ELF-1 and Mek4 in relation to the topographic development of the retinotectal projection. All the results from the RNA *in situ*, RAP *in situ*, and LAP *in situ* experiments appear internally consistent and support a role for the interaction of ELF-1 and Mek4. This is also consistent with the high affinity interaction of ELF-1 and Mek4 *in vitro* (an apparent K_D of approximately 10^{-9} M), at the high end of affinities so far reported for Eph ligands and receptors (Cheng and Flanagan, 1994). Most notably, the complementary gradients in expression and binding of ELF-1 and Mek4 appear consistent with Sperry's chemoaffinity theory predicting that position-specific information for topographic mapping in the retinotectal system should take the form of matching gradients of complementary cell-cell interaction molecules.

Studies of the cellular mechanisms that translate the retinotectal position-specific information into a map suggest a combination of effects on axon guidance, as well as on axon retraction and branching and possibly synapse formation and neurite survival (Walter et al., 1987; Nakamura and O'Leary, 1989; Boxberg et al., 1993; Roskies and O'Leary, 1994). ELF-1 might therefore affect one or more of these processes. The ligand and receptor gradients we detect here are in opposite orientations, with the highest point on the receptor gradient mapping to the lowest point on the ligand gradient and vice versa (Figure 7). A variety of possible mechanisms could account for mapping with gradients of this type. Since all the retinal axons enter the tectum at the anterior end and grow toward the posterior end, probably the simplest model would be a negative influence of ELF-1, acting to inhibit the progress or branching of temporal axons as they reach the posterior tectum. This model seems very compatible with *in vitro* assays that have shown topographically appropriate re-

sponses of retinal axons to tectal membranes. Assays of axon guidance (Walter et al., 1987), axon collapse (Cox et al., 1990), and axon branching (Roskies and O'Leary, 1994) have all detected an inhibitory activity that acts preferentially on temporal axons to cause axon repulsion, growth cone collapse, and inhibition of branching. Like ELF-1, all these activities are on tectal-cell membranes, all are at higher levels in posterior than in anterior tectum, and all are removed by PI-PLC treatment. In addition, posterior tectal membranes appear to have a positive activity that supports *in vitro* survival of neurites from nasal retina (Boxberg et al., 1993). Several recent studies provide precedents for molecules with inhibitory activities on axons, sometimes combined with positive effects on other axons (for example, Colamarino and Tessier-Lavigne, 1995; Mathes et al., 1995).

The properties of ELF-1 seem well suited to a role in transmitting accurate spatial information. The association of ELF-1 with cells of the tectum through a GPI anchor may be a critical feature ensuring that the spatial information it carries is stable and tightly localized. Also, the high concentrations of ELF-1 protein (Cheng and Flanagan, 1994) in the tectum may help in specifying a wide range of distinguishable position-specific values. Regarding the receptors in the retina, further work will be required to predict the molecular nature of the signal resulting from receptor occupancy, particularly because two receptors are present and ligand binding by cell-surface receptors does not necessarily result in receptor activation and can even result in inhibition of signaling (Eisenberg et al., 1990; Davis et al., 1994).

A critical feature of retinotectal mapping that models of topographic development have to explain is that the axons are arranged in a smooth and continuous map along the entire extent of each axis. Models with only one ligand and one receptor for each axis are possible, but they have the problem of explaining why all the axons do not project to one end of the target. It is also not easy for such models to account for size disparity experiments showing, for example, that after removal of parts of the retina the remaining axons can (under some circumstances) project to the topographically correct position, leaving other parts of the tectum uninervated (Sperry, 1963; Jacobson, 1991). Models with more than one receptor for each axis, or more than one ligand, could provide a way to account for continuous position-specific mapping, for example by guidance according to a difference between two signals. Since the Eph receptors and ligands are both families of closely related molecules, it seems plausible that other family members in addition to ELF-1 and Mek4 could be involved. In this regard, it is intriguing that Sek, a close relative of Mek4, is also expressed in retinal ganglion cells. Although Sek has a lower apparent affinity for ELF-1 and is not expressed in an obvious gradient, the presence of at least two receptors, both known to bind ELF-1, suggests models in which the decoding of the position-specific information might involve heterodimerization or differences in receptor binding or signaling properties.

ELF-1 could have other roles in nervous system development. The tectum contains multiple topographic maps,

including visual, auditory, somatosensory, and other spatial maps, which overlap in precise register and coordinate multimodal input and output according to its direction in external space (Udin and Fawcett, 1988; Holt and Harris, 1993). ELF-1 expression in the tectum could provide a simple mechanism for such maps to develop in an aligned manner. The patterns of *ELF-1*, *Mek4*, or *Sek* expression in other regions are also of interest; for example, expression in the hindbrain and spinal cord and in flanking areas such as the branchial arches, somites, and limb buds (Nieto et al., 1992; Cheng and Flanagan, 1994; Soans et al., 1994), suggests possible roles in patterning the cranial and spinal nerves.

Mek4 and *Sek* belong to the Eph receptor family, which is by far the largest known family of receptor tyrosine kinases. All of the receptors are closely related to one another, and almost all are known to be expressed prominently or exclusively in the nervous system. The identification of ELF-1 and other ligands now indicates that the Eph ligand family also forms a large group of closely related molecules. The recent evolutionary diversification of this large set of receptors and ligands could therefore be related to the acquisition of higher neural function in vertebrates, and during embryonic development these molecules could play a major role in the assembly of the precise and complex spatial order that characterizes the vertebrate nervous system.

Experimental Procedures

Identification of Chick *ELF-1* cDNA Clones

Chick *ELF-1* clones were isolated from a λ library of day 3 embryo cDNA by hybridizing with a mouse *ELF-1* probe (Cheng and Flanagan, 1994) and washing at low stringency ($2 \times$ SSC, 50°C). Two clones were characterized: *cE1-6* has a 2.0 kb insert and encodes a complete amino acid sequence for chick ELF-1, while *cE1-1* is truncated within the protein-coding sequence and has a 1.5 kb insert. The cDNA sequence has been deposited in the GenBank data base.

In Situ RNA Hybridization and Immunolocalization

Three separate antisense *ELF-1* probes were used for in situ hybridization. All gave similar results. One was a 1.0 kb fragment extending from the 5' end of *cE1-1* to a unique *AccI* site in the 3' untranslated sequence; the second was a 0.35 kb *StyI*-*AccI* fragment from the 3' untranslated region of *cE1-6*; and the third was a 0.6 kb fragment from the *AccI* site to the 3' end of *cE1-1*. *Mek4* and *Sek* RNA probes were from plasmids produced by PCR amplification of chick embryo cDNA: clone *cMek4HE* extends from a *HindIII* site at nucleotide 865 to an *EcoRI* site introduced at nucleotide 1651 of the chick *Mek4/Cek4* sequence (Sajjadi et al., 1991; GenBank accession number M68514). Clone *cSekHK* extends from a *HindIII* site at nucleotide 412 to a *KpnI* site at nucleotide 1222 of the chick *Sek/Cek8* sequence (Sajjadi and Pasquale, 1993; GenBank accession number Z19059).

In situ hybridization using digoxigenin-labeled RNA probes was performed as previously described (Wilkinson and Nieto, 1993; Cheng and Flanagan, 1994). Whole-mount analyses of *ELF-1* RNA were performed at days 2–4 of development on complete embryos or at days 6–11 on embryonic brain. For retinal sections, 20–24 μm frozen sections were prepared. For retinal whole mounts, the eye was dissected out and, after removal of the pigment epithelium, was fixed, hybridized, and stained, and then spread out for observation. For densitometry, whole retina or tectum was spread out on a filter, photographed on Kodak Ektachrome 160T film, digitized with a slide scanner, and profile plots were calculated using the NIH Image program.

Staining of Islet-1 was performed essentially as described elsewhere (Ericson et al., 1992; Tsuchida et al., 1994), using antibody 40.2D6, obtained through the Developmental Studies Hybridoma Bank at

Johns Hopkins University School of Medicine. For double detection, sections were first treated and developed for RNA staining and then for Islet-1 staining.

Production and Testing of ELF1-AP Fusion Protein

The *APtag-1* vector (Flanagan and Leder, 1990) was modified by transferring the *HindIII*-*XhoI* fragment with the polylinker and AP sequence into pcDNA1 (Invitrogen) to produce a new vector, *APtag-2*, which permits more rapid expression of fusion proteins by transient transfection in COS cells. Fusion protein concentrations are roughly comparable with stable transfections using *APtag-1*.

To produce an ELF1-AP fusion, *cE1-6* from nucleotide 34 in the 5' untranslated region to nucleotide 567 in the protein-coding sequence (GenBank accession number L40932) was amplified by PCR, adding terminal *HindIII* and *BglIII* sites, and inserted between the *HindIII* and *BglIII* sites of *APtag-2*, so Ser-177 of ELF-1 is fused to AP via a four amino acid linker (Arg-Ser-Ser-Gly). The resulting plasmid, pELF1S-AP, was transiently transfected with Lipofectamine (GIBCO BRL) into COS cells grown in DMEM with 10% bovine calf serum. Medium was changed 24 and 48 hr after transfection, and the supernatant was harvested after a further 4–6 days, as AP activity began to plateau. The supernatant was centrifuged, 0.45 μm filtered, and stored at 4°C with 20 mM HEPES (pH 7.0) and 0.05% sodium azide. The ELF1-AP concentration was approximately 3 $\mu\text{g}/\text{ml}$.

To test receptor binding by ELF1-AP, COS cells in six well dishes were transiently transfected with cDNAs for full-length mouse *Mek4* or *Sek* receptors (Sajjadi et al., 1991; Nieto et al., 1992) in the pcDNA1 vector, and cell-surface binding was tested as described previously (Cheng and Flanagan, 1994). For binding in a cell-free system, mouse *Mek4* extracellular domain, ending at His-540, was fused to an immunoglobulin tag (Aruffo et al., 1990), inserted in pcDNA1, and expressed in COS cells. *Mek4*-IgG fusion supernatant (0.5 ml) was incubated at room temperature for 1 hr on a rotator with 20 μl of protein A-agarose beads (Sigma), which were then washed three times with HBHA buffer (Hanks' balanced salt solution with 0.5 mg/ml BSA, 0.1% NaN_3 , 20 mM HEPES [pH 7.0]), incubated for 1 hr with ELF1-AP or AP control supernatant, washed three times with HBHA, washed three times with Triton-Tris (1% Triton X-100, 10 mM Tris-HCl [pH 8]), resuspended in 1 ml of Triton-Tris, and 10 μl aliquots were assayed colorimetrically for AP activity.

AP In Situ Analyses

Probes were mouse *Mek4*-AP or *Sek*-AP (Cheng and Flanagan, 1994) or chicken ELF1-AP. Whole mounts were performed on complete embryos at days 2–6 or on embryonic brain dissected out and freed of the pia mater at days 6–15. Tissues were treated with AP fusions unfixed, except for day 6 brain: because of its fragility, the embryos were fixed before dissection with 4% paraformaldehyde at room temperature for 90 min. Tissues were transferred to microfuge tubes or 24 well plates and then were treated with AP fusion supernatants as previously described (Cheng and Flanagan, 1994), followed by staining in BCIP and NBT for 5–15 min.

For explant cultures, temporal or nasal thirds of retinas were cut into 1 mm pieces and cultured as described previously (McLoon, 1991) for 36–48 hr on laminin-treated cover slides. Explants or frozen sections were rinsed with HBHA and then were overlaid with AP fusion supernatant for 75 min at room temperature, washed six times for 5 min each in HBHA, treated with acetone/formaldehyde fixative for 30 s, washed three times with HBS, and heated for 20–90 min at 65°C . Sections were stained for 4–6 hr, and explant cultures for 4–24 hr, with NBT and BCIP.

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