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# Shared and distinct functions of RAGS and ELF-1 in guiding retinal axons

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**Two ligands for Eph-related receptor tyrosine kinases, RAGS and ELF-1, have been implicated in the control of development of the retinotectal projection. Both molecules are expressed in overlapping gradients in the tectum, the target area of retinal ganglion cell axons. In two *in vitro* assays ELF-1 is shown to have a repellent axon guidance function for temporal, but apparently not for nasal axons. RAGS on the other hand is repellent for both types of axons, though to different degrees. Thus, RAGS and ELF-1 share some and differ in other properties. The biological activities of these molecules correlate with the strength of interaction with their receptors expressed on RGC axons. The meaning of these findings for guidance of retinal axons in the tectum is discussed.**

*Keywords*: axon guidance/Eph-related RTKs and ligands/gradients/retinotectal projection

## Introduction

The processes by which retinal ganglion cell axons find their correct position within the target area, the optic tectum, are a matter of long-standing investigation. The retinotectal projection represents a popular model system for the study of topographic projections, which are numerous in the nervous system and of central importance for brain functioning (Udin and Fawcett, 1988; Holt and Harris, 1993; Roskies *et al.*, 1995; Tessier-Lavigne, 1995; Goodman, 1996). The principle of topographic projections is to transfer faithfully spatially organized information from one group of neurons, the projecting area, onto another group of neurons, the target area.

A number of hypotheses have been put forward to explain the development of these projections involving either fibre–fibre interactions (Willshaw and Malsburg, 1979), time of arrival at the target (Rager, 1976) or synaptic stabilization due to functional validation (Willshaw and Malsburg, 1976; Whitelaw and Cowan, 1981). The currently most favoured hypothesis is based on the chemoaffinity theory proposed by Sperry some decades ago (Sperry, 1963). He suggested the presence of cytochemical tags on cells in both the projecting area and the target area. These tags would provide each cell with positional information, allowing a 'matching' of corresponding cells in the projecting and target areas, so that invading retinal axons carrying certain receptor molecules interact specifically with their corresponding counter-receptors on the tectum. He excluded a mosaic of many different molecules on both the retina and the tectum (mosaic theory), arguing that this would require too much genetic information as well as extensive random searching by invading axons for target positions, at least for misrouted fibres. Instead, he proposed quantitative differences, i.e. a graded expression of these cytochemical tags, meaning that positional information would be encoded in the form of relative amounts of a few molecules providing directional as well as positional cues. In order to specify internal target positions in the tectum, 'two antagonistic gradients—or at least two spatially antagonistic effects arising from the same graded distribution' (Gierer, 1988)—have been postulated for both the dorsoventral and nasotemporal axes, whereby the combined effect of these gradients leads to a local maximum or minimum of a guiding parameter (Gierer, 1983, 1988). For the retina, it is proposed that invading axon populations express cell surface receptors in a graded manner specifying the position of origin. In recent years, a number of molecules have been identified which are expressed in a graded manner in either the retina or the tectum, or both, such as $TOP_{AP}$ and $TOP_{DV}$ (Savitt *et al.*, 1995), TRAP (McLoon, 1991) and a 33 kDa protein (Stahl *et al.*, 1990).

In the retinotectal projection, axons from the temporal retina project to the anterior tectum, and axons from the nasal retina to the posterior tectum. In the perpendicular axis, dorsal retina is connected to ventral tectum and ventral retina to dorsal tectum (Mey and Thanos, 1992; Holt and Harris, 1993). *In vitro* assays established in recent years have provided an insight into the nature of guidance cues along the anteroposterior axis. In the stripe assay—where RGC axons are allowed to grow on alternating stripes of membranes from the anterior and posterior tectum—temporal axons are found to grow only on anterior membranes, thus reproducing the *in vivo* situation. Preference for anterior membranes was found to be due to repulsion by posterior membranes. The collapse assay (Cox *et al.*, 1990; Raper and Kapfhammer, 1990), in which retinal ganglion cells growing on a

laminin-coated surface are exposed to membrane fragments of interest and their behaviour is documented by time-lapse video microscopy, suggests that repulsion is due to collapse-inducing molecules in the posterior tectum. The repulsive molecules appear to be glycosylphosphatidylinositol (GPI)-anchored to membranes and have a graded distribution in the tectum, with maximal concentration in the posterior part.

These criteria were used to purify this activity and led to the cloning of a 25 kDa tectal protein named RAGS (for repellent axon guidance signal; Drescher *et al.*, 1995). RAGS emerged as a ligand for Eph-related receptor tyrosine kinases (for reviews, see Brambilla and Klein, 1995; Pandey *et al.*, 1995a). It was active in both the collapse and stripe assays, but had similar activity for temporal and nasal axons, in contradiction of its anticipated role, i.e. to be selectively repellent for temporal retinal axons. This was taken to indicate the existence of additional and/or modulatory activities conferring naso-temporal specificity (Drescher *et al.*, 1995).

Besides RAGS, another member of this family of Eph ligands, ELF-1 (for Eph ligand family-1), is known to be expressed in the tectum (Cheng and Flanagan, 1994). ELF-1 was cloned in a search for ligands of Eph-related receptors by using receptor alkaline phosphatase fusion proteins as probes. One of the receptors used, Mek4, and its corresponding ligand, ELF-1, were then found to be expressed in complementary gradients in the retina and tectum (Cheng *et al.*, 1995), in agreement with Sperry's concept.

Here, we show that ELF-1 and RAGS are expressed in the tectum in partially overlapping domains during the time of invasion of retinal axons. The present investigation concentrates on a comparative functional characterization of these two molecules. In a re-evaluation of RAGS function in the stripe assay, it transpires that this molecule can produce a concentration-dependent differential guidance of nasal and temporal axons. ELF-1, on the other hand, seems to have a bimodal effect in that it guides temporal axons but has apparently no effect on nasal axons. The activities of these molecules correlate with the strength of their interaction with the Cek4 receptor expressed on RGC axons. A preliminary model is presented that shows how RAGS and ELF-1 can account for the initial formation of the anteroposterior axis of the retinotectal projection.

## Results

### *Identification of GPI-anchored Eph-related ligands in the tectum*

In order to clone additional members of the family of ligands for Eph-related receptors expressed in the tectum, we used a cocktail of different probes derived from various Eph ligands for a low-stringency hybridization of a posterior tectum cDNA library (see Materials and methods). However, the only other GPI-anchored ligand different from RAGS was identified as ELF-1.

### *Comparative RNA expression analysis of RAGS and ELF-1*

To study the functional significance of the simultaneous expression of two closely related Eph ligands in the tectum, we performed a detailed RNA expression analysis of both molecules at various developmental stages using DIG-labelled RNA probes (Figure 1A–E).

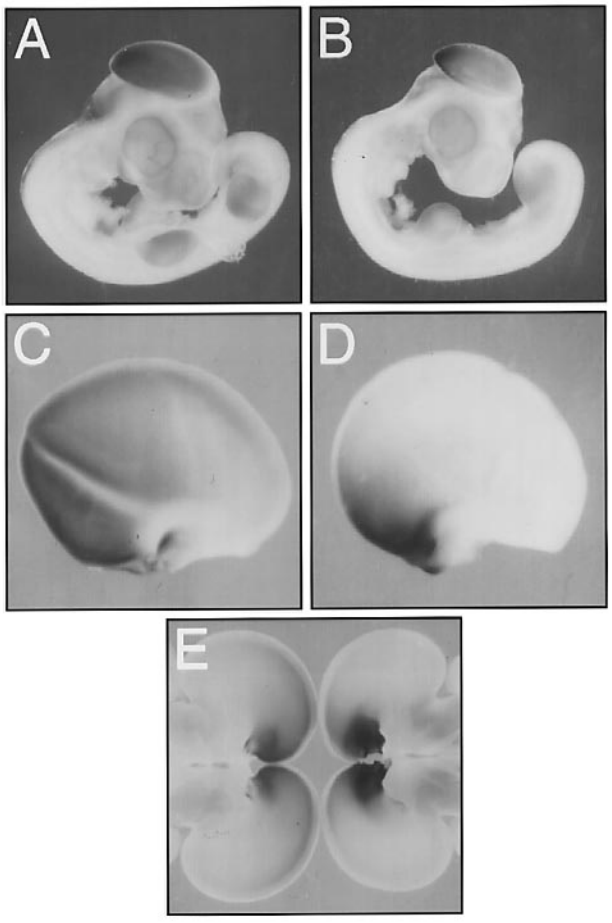



**Fig. 1.** RNA expression of RAGS and ELF-1 in the developing visual system. (A–E) Whole mounts or isolated tecta were hybridized with ELF-1- (A, C and E) and RAGS- (B, D and E) specific DIG-labelled antisense probes. (**A** and **B**) Day 4 embryo, viewed laterally; (**C** and **D**) day 7 embryo, viewed ventrally; posterior is to the left. (**E**) Day 9 embryo, viewed dorsally; the tecta on the left side were hybridized with an ELF-1-specific probe, those on the right side with a RAGS-specific probe. Posterior poles of the tecta are oriented toward the centre of the figure. Colour reactions were stopped usually after ~1.5 h for ELF-1 and ~6 h for RAGS. Prolonged reaction times do not lead to an obvious staining of the anterior tectum for RAGS, while for ELF-1 the whole tectum is stained. Owing to differences in GC content the ELF-1 probe was found to be ~8-fold more sensitive than the RAGS probe (see Materials and methods).

At all time points analysed, RAGS and ELF-1 RNAs are expressed in gradients, with higher expression in the posterior part of the tectum. The ELF-1 expression domain at E4 covers the entire tectum (Figure 1A), whereas later expression in the anterior part seems to be reduced (Figure 1C and E). In contrast, the expression domain of RAGS is generally found more restricted to the posterior part of the tectum throughout the developmental time analysed (Figure 1B, D and E). A very strong expression at the posterior pole is apparent.

In summary, both RAGS and ELF-1 are expressed in gradients in the tectum, but the RAGS gradient seems to be steeper and more confined to the posterior part of the tectum than ELF-1.

### *Localization of RAGS and ELF-1 protein in the developing tectum*

If RAGS and ELF-1 function as axon guidance molecules, then retinal axons should co-localize with RAGS and ELF-1 protein during their ingrowth through the superficial layers of the tectum. RNA coding for RAGS is primarily located not in superficial, but in ventricular inner cell layers (Drescher *et al.*, 1995). These layers contain radial glial cells possessing processes which span the tectum, ending in endfeet on the surface of the tectum. To localize RAGS and ELF-1 protein, an immunohistochemical analysis was performed using monoclonal antibodies specific for these two ligands (see Materials and methods). As shown in Figure 2B, RAGS protein can be detected not only in ventricular, but also in other layers of the tectum, including the superficial layers. At higher magnification, staining of processes which span the tectum from inner to outer layers can be identified (Figure 2E). This suggests that part of the observed expression pattern of RAGS protein reflects a process in which this ligand is produced in radial glial cells and then transported into the endfeet at the surface of the tectum, which is in contact with invading retinal axons. RAGS protein should also be expressed in other cell types, as corresponding RNA (at lower levels) can also be found in intermediate layers (Drescher *et al.*, 1995). It is also conceivable that part of the observed protein distribution reflects other mechanisms such as migration of RAGS-expressing cells from inner to more superficial layers of the tectum.

ELF-1 protein can be detected in similar locations to RAGS and is therefore also accessible to contact by ingrowing axons (Figure 2A). A quantification of RAGS and ELF-1 immunofluorescence staining (shown in Figure 2C and D) is consistent with the corresponding RNA expression data (Figure 1), in that the gradient of RAGS protein appears to be steeper and more confined to the posterior part of the tectum compared with the ELF-1 expression pattern.

### *Binding of RAGS and ELF-1 to Eph-related receptors expressed in the retina*

We set out to identify the cytochemical tags on RGC axons corresponding to these ligands. A characteristic of the Eph-related family is the promiscuity in the interaction of receptors and both GPI-anchored (Cheng and Flanagan, 1994; Davis *et al.*, 1994; Kozlosky *et al.*, 1995) and transmembrane ligands (Bergemann *et al.*, 1995; Brambilla *et al.*, 1995), which might also hold true for the two ligands RAGS and ELF-1. Therefore, we focused on Eph-related RTKs which are believed to interact specifically with GPI-anchored ligands, namely Eck (Lindberg and Hunter, 1990), Cek4 (Sajjadi *et al.*, 1991), Cek7 (Siever and Verderame, 1994) and Cek8 (Sajjadi and Pasquale, 1993). As the Eck receptor is not expressed at relevant times in the visual system (Ganju *et al.*, 1994; Ruiz and Robertson, 1994), we concentrated on the latter three.

Cek7 was the prime candidate for the relevant RAGS receptor, as it was shown that a species homologue of this receptor, Rek7, interacts specifically with the human homologue of RAGS, AL-1 (Winslow *et al.*, 1995). An immunohistochemical analysis performed between E9 and E13 showed expression of Cek7 in various layers of the retina, including the RGC layer, with no obvious gradient

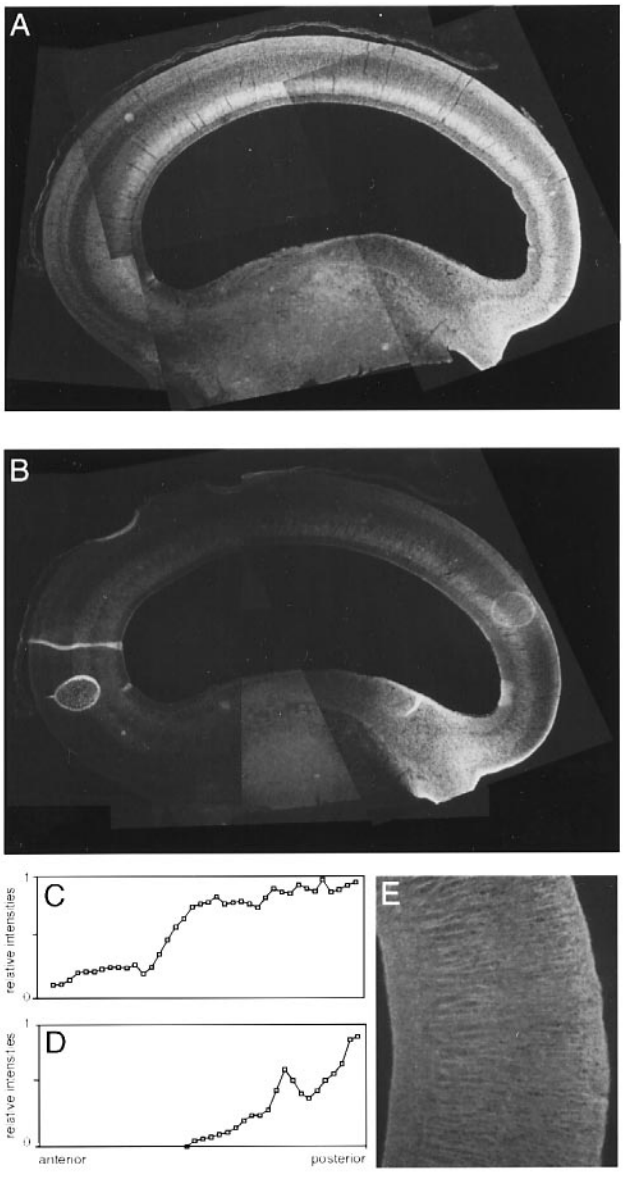



**Fig. 2.** Expression of RAGS and ELF-1 protein in the developing tectum. Immunohistochemical analysis of the expression pattern of Eph ligands in para-sagittal sections of E9 chicken tecta using monoclonal antibodies specific for ELF-1 (**A**) and RAGS (**B**). Composite pictures are shown. In each case pictures were taken under manual control with the same exposure times (RAGS: 10 s; ELF-1: 5 s). Anterior is to the left, posterior to the right. (**C** and **D**) Measurements of fluorescence intensity of the outer surface (top) of these tecta (see Materials and methods). (**E**) Magnification of a staining of an E9 posterior tectal section with a RAGS-specific monoclonal antibody.

along the anteroposterior axis (D.A.Siever and M.F. Verderame, manuscript in preparation). As shown by Cheng *et al.* (1995), Cek4 and Cek8 are expressed at E8 in the retina. Cek4 is expressed differentially in the RGC layer, with higher expression in the temporal half than in the nasal half, while Cek8 is expressed uniformly. However, on the basis of Northern blot analyses from E7 retina, Cek8 RNA seems to be slightly more abundant in the nasal half of the retina (data not shown). This finding correlates with the time of differentiation of retinal ganglion cells of temporal and nasal retina (Rager *et al.*, 1993).

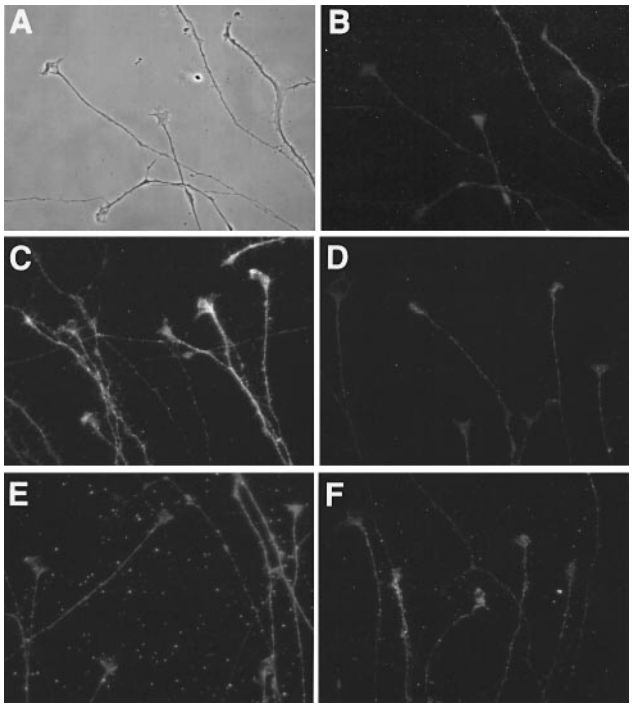



**Fig. 3.** Expression of Cek4, Cek7 and Cek8 on E6 retinal ganglion cell axons and growth cones. Axons grown from retinal explants *in vitro* were immunostained with (**C** and **D**) Cek4, (**E**) Cek7 and (**F**) Cek8 antisera. Controls were done without primary antibody, visualized by (**A**) phase-contrast and (**B**) fluorescence microscopy. Cek4 staining was stronger on axons grown from (C) temporal compared with (D) nasal retinal explants. No such difference was seen for Cek7 and Cek8 staining.

All three receptors were shown immunohistochemically, by using specific antibodies, to be located on RGC axons (Figure 3). An analysis of the binding affinities of these three receptors to RAGS and ELF-1 is therefore essential for dissecting their biological function.

For a precise quantification of binding affinities, the receptor alkaline phosphatase (RAP) technique (Flanagan and Leder, 1990) was used. Various fusion proteins containing the extracellular domain of individual receptors linked to the coding region of alkaline phosphatase (AP) were generated. These are soluble tags and were used to probe Cos cells expressing the ligands. Dissociation constants for receptor–ligand pairs were then determined on the basis of a Scatchard analysis (Scatchard, 1949).

As shown in Figure 4 and illustrated diagrammatically in Figure 5, the strongest interaction was seen between Cek4 and RAGS, with a dissociation constant of $1.44\times10^{-10}$ M. ELF-1, in contrast, bound to Cek4 with a $K_d$ of $8.60\times10^{-10}$ M. The interaction of ELF-1 with Cek4 was in the same range as the interaction of RAGS with the Cek7 and Cek8 receptors ($6.16\times10^{-10}$ M and $6.22\times10^{-10}$ M). The interaction of ELF-1 with Cek7 and Cek8 was weakest with $K_d$s of $8.62\times10^{-9}$ M and $1.27\times10^{-8}$ M, respectively. In summary, three different categories of interactions with respect to $K_d$ values are evident: a very strong binding of RAGS to Cek4, a strong interaction of RAGS with Cek7 and Cek8, very similar to the binding of ELF-1 to Cek4, and a weak binding of ELF-1 to Cek7 and Cek8.

In a further investigation of the interaction between receptors and ligands, it could be shown that ELF-1 and RAGS can induce the phosphorylation of both Cek4 and Cek8 (Ohta *et al.*, 1996; K.Ohta, H.Iwamasa, U.Drescher, H.Terasaki and H.Tanaka, manuscript in preparation), as is true for Cek7 (Shao *et al.*, 1995; Winslow *et al.*, 1995).

Very recent studies by Gale *et al.* (1996) have shown that RAGS and ELF-1 can also bind to the Eph-related receptors Ehk-2 (Maisonpierre *et al.*, 1993) and Ehk3/Mdk1 (Ciossek *et al.*, 1995; Valenzuela *et al.*, 1995). Further investigations will be directed toward a possible expression of these receptors in the retinotectal system.

### *Comparative functional analysis of ELF-1 and RAGS*

The expression of ELF-1, a member of the same ligand family as RAGS, in the tectum and its interaction with the same set of RGC-expressed, Eph-related receptors as RAGS suggests an involvement of this molecule in the formation of the retinotectal projection. To investigate this, an *elf-1* cDNA-containing expression plasmid was transfected into Cos cells; 2 days later membranes from these cells were isolated and analysed in the stripe assay. These membranes were prepared in alternating lanes with mock-transfected Cos cell membranes. Strikingly, in this set of experiments, temporal axons avoided ELF-1-containing Cos cell membranes, while nasal axons grew equally well on both types of membranes, indicating a very clear *in vitro* guidance activity of ELF-1 for temporal but, at least under these experimental conditions, not for nasal axons (Figure 6A). This clear-cut difference in axon guidance is comparable with that seen in 'standard' stripe assays using anterior and posterior tectal membranes (Walter *et al.*, 1987).

To reinforce conclusions from the stripe assay, ELF-1 function was analysed in the collapse assay. ELF-1-containing membranes from transiently transfected Cos cells elicited only a weak response in the collapse assay (data not shown), whereas membranes derived from the same transfection led to a guidance of temporal axons in the stripe assay (see above). This result suggests that the stripe assay is more sensitive in detecting molecules with a potential axon guidance activity than is the collapse assay. In making this comparison it is assumed that both assays detect mechanistically similar activities (Walter *et al.*, 1990; Fan and Raper, 1995).

To increase the relative amounts of ELF-1 in the relevant membrane fractions, we established human 293 cell lines stably expressing high amounts of ELF-1. As estimated from a rough quantification of ELF-1 by determining Cek4-AP binding activity (see Materials and methods), membranes from selected cell lines contain ~8-fold higher concentrations of ELF-1 than do transiently transfected Cos cells. With membranes containing higher amounts of ELF-1, a strong collapse-inducing activity was detected. As in the stripe assay, this transpired to be specific for temporal RGC growth cones. Here, 5 μg of 293/ELF-1 membranes induced 100% collapse of temporal (34/34), but only 16.7% collapse of nasal growth cones (4/24). In control experiments using the same amount of mock-transfected 293 cell membranes, retinal growth cones were barely affected [temporal growth cones: 6.3% (2/32); nasal growth cones: 14.8% (4/27)]. Even with very high amounts of ELF-1 membranes, no effect on nasal growth cones was seen. This indicates a broad concentration range in

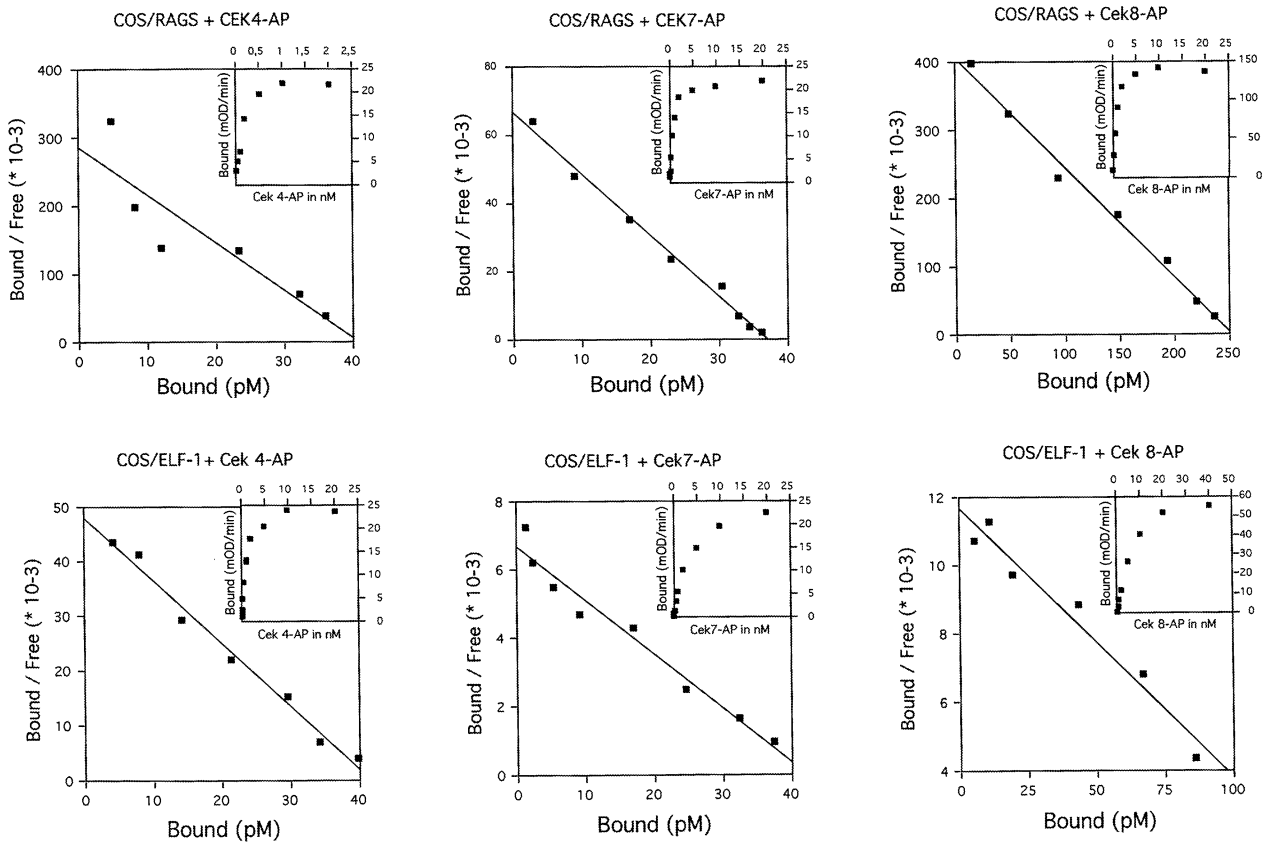



**Fig. 4.** Binding of RAGS and ELF-1 to Eph-related receptors. Scatchard analysis of the binding of Cek4-AP, Cek7-AP and Cek8-AP to membrane-bound RAGS and ELF-1. Hyperbolic representations are shown as insets. The binding characteristics calculated from these experiments are shown schematically in Figure 5.

which ELF-1 shows a bimodal effect on temporal versus nasal axons. Further experiments using still higher amounts of membranes were not carried out because they caused severe non-specific growth cone collapse.

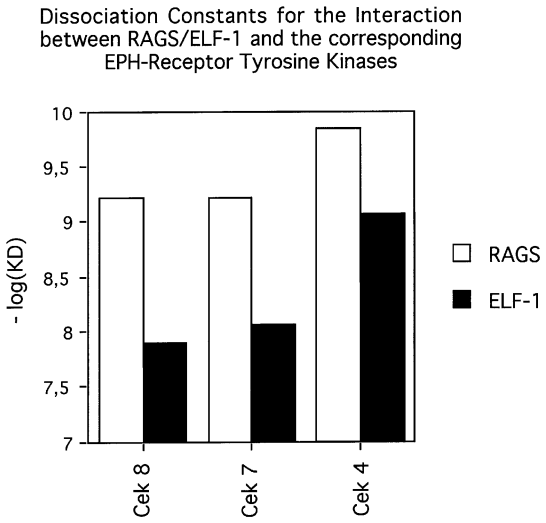
The interaction of RAGS with the relevant Eph-related receptors expressed on RGC axons led us to functionally re-characterize RAGS itself. RAGS binds with high affinity to all three receptors, but owing to differences in binding affinity ($K_d$ $1.44\times10^{-10}$ for Cek4, $K_d$ $6.16\times10^{-10}$ for Cek7, $K_d$ $6.22\times10^{-10}$ for Cek8; Figures 4 and 5) it should be expected that at higher concentrations all three receptors will be activated, but at lower concentrations the Cek4 receptor, which is expressed more strongly on temporal axons, will be preferentially activated.

In the collapse assay, it became apparent that RAGS at 10 μg of total membrane protein shows growth cone collapse-inducing activity with little topographic specificity (Figure 7). However, as predicted from the biochemical data, at lower amounts of membranes (e.g. 3 μg), temporal and nasal growth cones showed a distinct difference in their sensitivity to RAGS. Even at these low amounts of membrane, 50% of temporal growth cones collapsed, while nasal axons were no longer affected (Figure 7).

These functional characteristics of RAGS were also apparent in stripe assay analyses (Figure 6B and C). Undiluted RAGS-containing Cos cell membranes derived from a transient transfection led to a very strong repulsion of both nasal and temporal axons, concomitant with a striped pattern of axon growth (Drescher *et al.*, 1995). A stepwise 2-fold dilution series of the RAGS membranes resulted in a reduction of the strength of the growth decision of nasal and temporal axons. With successive dilutions, nasal axons are affected first, losing their striped growth pattern (Figure 6B), whereas temporal axons become insensitive only at higher dilutions of RAGS (Figure 6C). The range of transition from striped to non-striped growth behaviour could be shifted toward either the temporal or the nasal side of the retinal explant by varying the dilution of RAGS-containing Cos cell membranes.

## Discussion

The present study represents a comparative biochemical and functional characterization of RAGS and ELF-1, two Eph-related ligands expressed in gradients in the developing chicken tectum, and the interaction with their putative receptors expressed on retinal axons. From this *in vitro* study it can be inferred that these molecules are major players in determining the positioning of terminal arborizations with respect to the anteroposterior axis and are good candidates for the cytochemical tags proposed by Sperry (1963). In the stripe assay, it transpires that RAGS and ELF-1 possess shared and also unique features. ELF-1 is a repellent axon guidance molecule for temporal axons with apparently no effect on nasal axons. RAGS




| EPH-Receptor | $K_D$ for RAGS | $K_D$ for ELF-1 |
|---|---|---|
| Cek 8 | $6{,}22 \times 10^{-10}$ | $1{,}27 \times 10^{-8}$ |
| Cek 7 | $6{,}16 \times 10^{-10}$ | $8{,}62 \times 10^{-9}$ |
| Cek 4 | $1{,}44 \times 10^{-10}$ | $8{,}60 \times 10^{-10}$ |

**Fig. 5.** Logarithmic representation of dissociation constants for Cek4-AP, Cek7-AP, and Cek8-AP, and RAGS and ELF-1. After regression analysis of the binding data shown in Figure 4, the negative reciprocal slope from the Scatchard equation was taken as the dissociation constant.

repels both types of retinal axon, with temporal axons being more sensitive than nasal ones.

Although the function of these molecules *in vivo* remains unclear, data provided by the stripe assay might prove a good basis for making predictions about such function, since this assay may closely resemble conditions found *in vivo*. Furthermore, the combination of these data with the spatial expression patterns of RAGS, ELF-1 and their putative receptors, as well as the biochemical characteristics of the interaction between these ligands and receptors, produces a preliminary model of the involvement of these molecules in the formation of the chick retinotectal projection.

Based on the present data, we propose that ELF-1 is a molecule which functions *in vivo* to keep temporal axons from invading the posterior tectum. ELF-1 and RAGS are both expressed in the posterior tectum. As the expression domain of ELF-1 extends more anteriorly than that of RAGS, invading retinal axons are confronted first with ELF-1 rather than RAGS. It is likely the differential expression of the Cek4 receptor on these axons which gives rise to the differential guidance of temporal versus nasal axons in the anterior tectum. As inferred from the stripe assay, this interaction results in the repulsion of temporal axons through the activation of Cek4; on the other hand, nasal axons are not affected. We hypothesize that they are able to grow toward the posterior tectum because they express lower concentrations of the Cek4 receptor than do temporal axons. The observation that ELF-1 has no effect on nasal axons in either in the collapse assay or the stripe assay indicates that the interaction of ELF-1 with Cek7 and Cek8 is not involved in axon repulsion. However, due to experimental constraints we cannot exclude that, at very high concentrations, nasal axons are also affected by ELF-1.

Any prediction of the *in vivo* function of RAGS is difficult because its concentration in the tectum is currently unknown. RAGS is not expressed in the more anterior part of the tectum but is, like ELF-1, expressed in a graded manner in the posterior part. In the preliminary model proposed here, RAGS interacts in this area with axons which have surmounted the gradient of repellent activity of ELF-1 in the anterior tectum. This would be the case for nasal axons, for which Cek4 receptor concentration and activation by ELF-1 is no longer sufficient to induce a repulsion. RAGS has a higher affinity for the Cek4 receptor than ELF-1. Indeed, the concentration-dependent transition from a guided to a non-guided growth of nasal axons seen in stripe assays using RAGS-containing membranes fits nicely with a concept in which RAGS interacts with a receptor that is expressed differentially along the nasotemporal axis of the retina, a pattern matching the expression of Cek4. Inferred from expression patterns of the two ligands, one might hypothesize that, *in vivo*, the repulsion of nasal axons is dependent on the concerted activation of Cek4 by RAGS and ELF-1. In this context, RAGS might be regarded as a high-affinity repellent and ELF-1 as a low-affinity repellent.

The role of the Cek7 and Cek8 receptors in formation of the retinotectal projection is unknown at present and requires further investigation. Nevertheless, according to binding affinities (Figures 4 and 5) it is conceivable that RAGS, but not ELF-1, can induce a strong activation of both receptors. Therefore, it would be the sum of signals from possibly all three receptors which is integrated by the growth cones and is the basis for repulsion of nasal axons in the posterior tectum. However, our lack of knowledge of the function of Cek7 and Cek8 receptors and the fact that other Eph-related receptors and ligands are expressed during development of the retinotectal projection (Kenny *et al.*, 1995; Holash and Pasquale, 1995; U.Drescher, unpublished results) underscore the preliminary nature of the presented model and point to a complex mechanism for control of this projection.

This preliminary model also does not provide an explanation for the abrupt transition of retinal axons from guided to non-guided growth typically seen in stripe assays using anterior/posterior tectal membranes and ELF-1-containing membranes. In general, areas in which sharp transitions occur are often interpreted as zones where a threshold value of one graded parameter is transformed into an abrupt change of another parameter. It would indicate that, at the border between nasal and temporal axons, the activation of Cek4 receptors by ELF-1 has reached such a threshold value, resulting in an abrupt change in the guidance behaviour. However, this phenomenon could likewise be explained by, for example, an antagonstic effect of an as yet unidentified receptor (see above) or a modulation of the receptor-activated signal transduction pathways.

Although it seems only natural to predict that temporal and nasal axons are guided to their proper targets on the

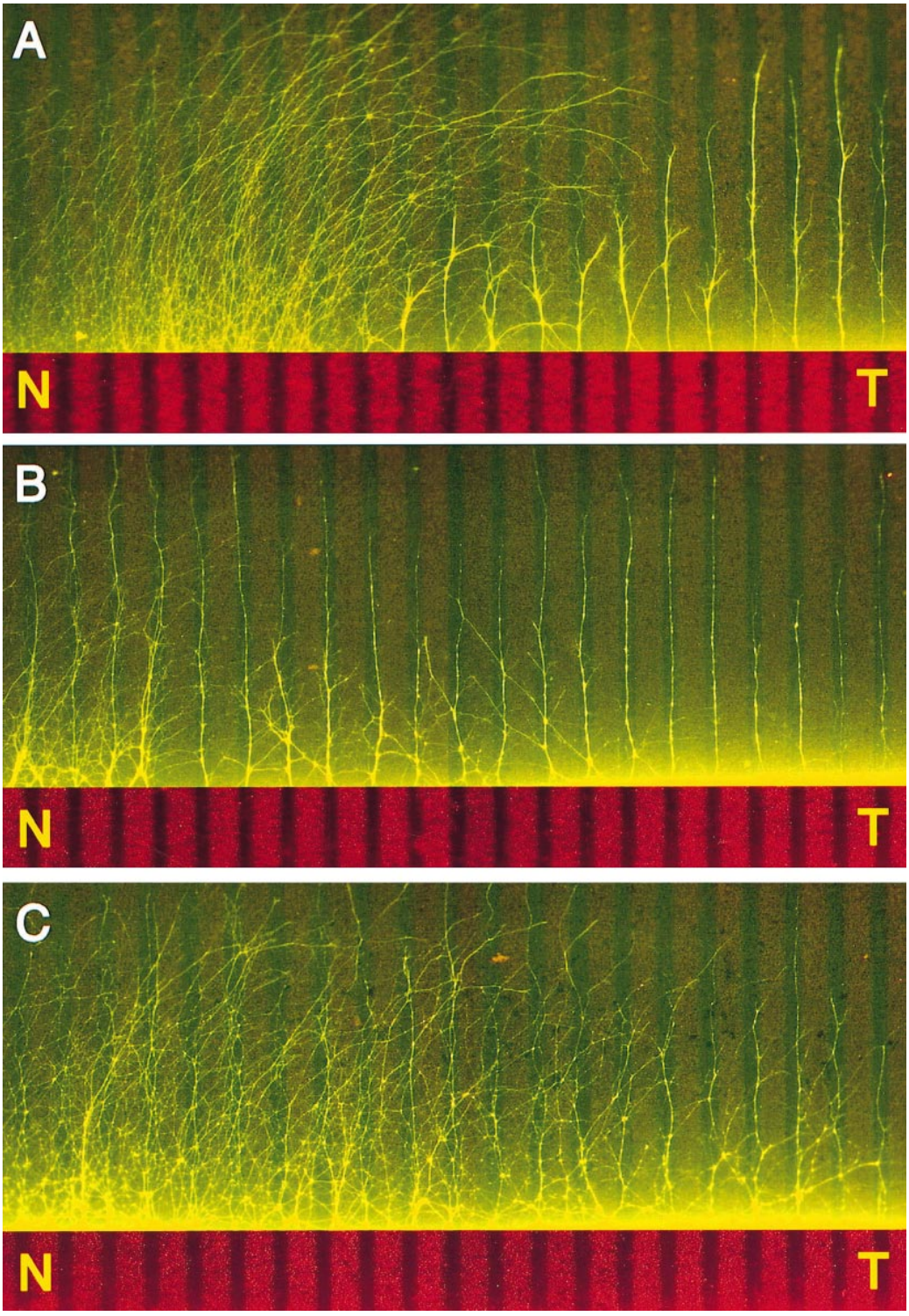



**Fig. 6.** Functional characterization of ELF-1 and RAGS in the stripe assay. Nasal axons (N) and temporal axons (T) were given the choice of growing on membranes derived either from mock-transfected Cos cells or from Cos cells transfected with (**A**) ELF-1 or (**B** and **C**) RAGS. The ELF-1/RAGS-transfected cell membranes are labelled with rhodamine isothiocyanate (RITC) fluorescent beads, visualized in the lower part of each figure. RAGS-transfected Cos cells were used at dilutions of 1:2 (B) and 1:4 (C). Retinal ganglion cells were stained with DiAsp during preparation of retinal tissue.

tectum by principally the same mechanisms, the question remains as to why a repellent guidance effect has never been observed in the stripe assay on nasal axons using native posterior tectal membranes, even though there is with RAGS a molecule expressed in the tectum which has, at least *in vitro*, a repellent activity on this type of axon. Besides other possibilities, one simple answer would be that RAGS is diluted during preparation of posterior tectal membranes to such an extent that its concentration is no longer sufficient for a repulsion of nasal axons.

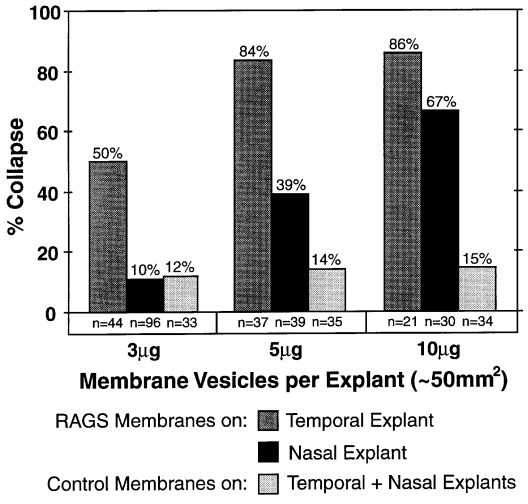



**Fig. 7.** Growth cone collapse-inducing activity of RAGS-transfected Cos cell membranes for nasal and temporal retinal ganglion cells. Different amounts of Cos cell membrane vesicles, quantified by determining the total protein content (μg), were tested on growth cones from temporal and nasal retinal explants.

The similarity in function of RAGS and ELF-1 is reflected in their sequence similarity, which is the highest within the GPI-anchored Eph ligand family (Drescher *et al.*, 1995). It indicates that both genes arose by duplication of a common ancestral gene with subsequent diversification in expression domains and functions. Driving forces for such a duplication might be evolutionary constraints. It is speculated that a growth cone needs a certain steepness in gradient of a guidance molecule for proper orientation in its target area. Beyond a certain size of this target area, the graded expression of only one such molecule would no longer be sufficient. The proper encoding of positional information could be achieved by means of the overlapping expression pattern of two guidance molecules with different binding affinities to relevant receptors expressed on invading axons.

Functionally, the Eph family has also been analysed in more distant systems, e.g. in angiogenesis, where B61/Lerk1, another ligand of Eph receptors, was shown to be involved in the migration of endothelial cells (Pandey *et al.*, 1995b), in hindbrain segmentation (Lumsden, 1990), where for example sek1 was implicated in the control of rhombomere formation (Xu *et al.*, 1996a), and recently also in patterning the developing forebrain (Xu *et al.*, 1996b), possibly through restriction of cell migration. As axon guidance can be regarded as a form of cell migration (Pfenninger, 1986; Keynes and Cook, 1990; Jesuthasan, 1996), it is perhaps not surprising that the Eph family encodes positional tags for both migrating cells and growth cones. Moreover, although the expression domains of certain Eph ligands seem thus far to represent 'forbidden' territory for axons expressing complementary receptors, this family could well, like the netrins (Colamarino and Tessier-Lavigne, 1995), also have attractive aspects.

## Materials and methods

### *Cloning of cDNAs coding for ligands and receptors of the Eph family*

For a low-stringency cDNA library screen, three different mouse ligands for the Eph-related receptor tyrosine kinase family were cloned by RT–PCR and used along with RAGS as probes in a low-stringency cDNA library screen. The coding regions of ELF-1 (Cheng and Flanagan, 1994), Lerk-2 (Beckmann *et al.*, 1994) and Htk ligand (Bennett *et al.*, 1995) were PCR-amplified from reverse-transcribed mouse P0 midbrain RNA and cloned into pBluescript II KS+ (Stratagene). The insert fragments were used as templates to generate $^{32}$P-labelled probes (T7 Quick Prime Kit, Pharmacia). In order to clone additional chick ligands, a randomly primed cDNA library from chicken E8 posterior tectum poly(A)$^+$ RNA was used (Drescher *et al.*, 1995). Approximately 500 000 clones of an amplified version of this cDNA library were screened with a cocktail of all three mouse probes and a RAGS probe. Hybridization to filters was done in 6× SSC, 5× Denhardt's, 0.5% SDS, 100 μg/ml salmon sperm DNA at 65°C overnight. Filters were washed finally in 1× SSC, 0.2% SDS at 55°C. Twenty-six ELF-1 positive clones were obtained, replated and rescreened to obtain individual clones. The 910 bp insert of clone 21/2b was found to include the entire sequence of a 665 bp full-length chick ELF-1 clone, published by Cheng *et al.* (1995).

For isolation of a full-length Cek4 cDNA clone, a λ zap cDNA library made from chicken retina E5 was screened under standard conditions using a mixture of fragments covering the highly conserved kinase domain of Eph-related receptors. Individual phage plaques were re-screened twice to obtain individual phages. To identify cDNA clones encoding Cek4, phage plaques were probed with a Cek4-specific PCR fragment (nucleotides 561–793; Sajjadi *et al.*, 1991). Following sequencing and restriction mapping, one clone was found to contain the full-length coding region of Cek4.

### *Whole-mount in situ RNA hybridization*

The full-length cDNA of chick ELF-1 clone 21/2b was subcloned into pBluescript II KS+ (Stratagene) from which the multiple cloning site had been deleted with *Apa*I and *Sac*II. After linearization by cutting internally with *Pst*I, a digoxigenin-11-UTP (DIG)-labelled antisense RNA probe covering 70 bp of coding region and 251 bp of 3′UTR was generated by *in vitro* transcription. The subcloned full-length RAGS cDNA (pBluescript II KS+) was linearized by cutting internally with *Pvu*II and *in vitro* transcribed to yield an RNA probe covering 201 bp of coding region and 87 bp of 3′UTR. A dot-blot analysis of various dilutions of *in vitro*-transcribed RAGS and ELF-1 sense RNA, hybridized with these DIG-labelled antisense probes, revealed an ~8-fold higher sensitivity for the ELF-1 probe (68% GC content) compared with the RAGS probe (46.2% GC content). Whole-mount *in situ* hybridizations were performed at embryonic days 4, 7 and 9 following a protocol by Wilkinson (1995). In brief, the tissue is fixed overnight in 4% paraformaldehyde/PBS. After dehydration/rehydration in a methanol series the tissue is bleached for 1 h in 6% $H_2O_2$. A proteinase K step (10 μg/ml) at room temperature is followed by postfixation in 4% paraformaldehyde/0.2% glutaraldehyde for 20 min. Hybridization is done in 50% formamide, 5× SSC pH 4.5, 50 μg/ml yeast RNA, 1% SDS, 50 μg/ml heparin at 70°C overnight. After washing twice at 70°C for 30 min in formamide, 5× SSC/1% SDS, an RNase step is included (2×30 min at 37°C with 100 μg/ml). After washing three times for 30 min at 65°C in formamide/2× SSC and a blocking step in 10% normal goat serum in TBST, the tissue is incubated with anti-DIG antibody overnight at 4°C. The following day and overnight the tissue is washed extensively in TBST, and thereafter developed in NTMT plus NBT/BCIP for the indicated times.

### *Immunostaining*

Chick E6 retinal explants were grown overnight on a poly-D-lysine/laminin-coated glass coverslip in F12 culture medium. After fixation for 15 min in 4% paraformaldehyde the cultures were permeabilized by treating with cold methanol at –20°C for 10 min. The cultures were stained with rabbit polyclonal antibodies to Cek4, Cek7 and Cek8. Anti-Cek4 and anti-Cek8 were affinity-purified antibodies to the carboxy-terminal peptide of the respective kinases and were used at a concentration of 10–20 μg/ml (Holash and Pasquale, 1995). Anti-Cek7 serum (sp682) was obtained by immunizing with a Cek7 juxtamembrane region peptide and was used in a 1:100 dilution. After overnight incubation at 4°C, the primary antibody binding was visualized using a biotin-labelled secondary antibody and streptavidin-Cy3 (Dianova).

Monoclonal antibodies specific for RAGS and ELF-1 were generated following established protocols (Harlow and Lane, 1988). BALB/c female mice were immunized in 2-week intervals with 100 μg RAGS-$F_c$ or ELF-1–$F_c$ fusion proteins (K.Ohta, T.Kaneko, T.Yamada and H.Tanaka, manuscript in preparation). Monoclonal antibodies used in cryostat sections of tecta were characterized by staining of Cos and 293 cells transfected either with RAGS or ELF-1 cDNA-containing

expression vectors. In these cases monoclonal antibodies bound only to the correspondingly transfected cells with no cross-reactivity to the inappropriate ligand. Standard SDS–PAGE of E6 chicken tecta as well as of transfected Cos and 293 cells under reducing conditions followed by Western blot analyses were not successful, indicating that the monoclonal antibodies used recognize native epitopes only. However, in subsequent analyses under non-reducing conditions, only protein bands of the appropriate size were identified.

For immunofluorescence measurements, tecta were fixed in 4% paraformaldehyde and, after overnight incubation with the monoclonal antibodies at 4°C, biotin-labelled IgG-specific secondary antibody and streptavidin-Cy3 were used for visualizing RAGS and ELF-1 protein. Measurements of fluorescence staining shown in Figure 2C and D were made using the NIH Image program, in which the intensities of adjacent rectangles covering the surface of the tectum were determined. For these analyses the individual pictures and not the composite pictures shown in Figure 2A and B were used. Subsequently, the resulting graphs were aligned on the basis of fixed points on the tectum.

### *Construction and expression of alkaline phosphatase fusion proteins*

To generate AP fusion proteins of Cek4, 7 and 8, the cDNA sequences corresponding to the extracellular domains were amplified by PCR and cloned into CMV-AP, a derivative of the AP-tag-1 vector (Flanagan and Leder, 1990). CMV-AP was constructed by excising the MoMLV promoter via *Sna*BI and replacing it by a blunt-ended *Eco*RI–*Sma*I fragment of the CMV promoter from plasmid CMVβ-gal (Clontech). In detail, for Cek4 primers were 5′-TTTAGATCTTGCCCCGCCGACA-TGGA-3′ as the upstream primer containing the endogenous ATG start codon and 5′-TTTTCCGGACTGGCTATTTTCACTGGAAA-3′ as the downstream primer, thereby covering the extracellular domain of Cek4 from nucleotides 1 to 1651 (Sajjadi *et al.*, 1991). For Cek 7 (clone 9/11; Siever and Verderame, 1994), a 481 bp fragment from nucleotides 1244–1725 was PCR-amplified using 5′-5′-ATGTCAGGTACCTCCCCCA-GCAA-3′ and 5′-AAAGATCTCTGGCTCTGGTCACTGGATGCAG-3′ as primers and cloned 3′ to the *Kpn*I site at position 1250 of the *Eco*RI–*Kpn*I fragment of Cek7 (nucleotides 1–1250). The entire extracellular domain from 1 to 1725 was then excised by *Eco*RI and *Sac*I and blunt-end cloned into the *Bgl*II site of CMV-AP. Nucleotides 1–1642 comprising the extracellular domain of Cek 8 (Ohta *et al.*, 1996) were amplified using primers 5′-TTTAGATCTGGTATCGATAAGCTTGATATC-3′ and 5′-TTTAGATCTTCTGGTTTAGATCTGGTATCGATAAGCTTGATATC-3 and 5′-TTTAGATCTTCTGGGATTGGTACCATCGC-3′. The cDNA fragment was cloned into CMV-AP via the *Bgl*II site. For transient expression of CekX–AP fusion proteins, the corresponding cDNAs were transfected into Cos cells by calcium phosphate precipitation (Chen and Okayama, 1987). The cells were grown for 6–8 days until AP activity in the supernatant reached its maximum. The enzymatic activity of the fusion proteins was measured according to Flanagan and Leder (1990) in a Shimadzu UV-160A spectrophotometer. Supernatants could be stored for a few months after addition of 20 mM HEPES pH 7.0 and 0.05% sodium azide (final concentration).

### *Quantitative binding of CekX–AP fusion proteins to ligand-expressing cells*

CekX–AP fusion protein-containing cell supernatants were concentrated in a 200 ml Amicon ultrafiltration cell through a XM 50 kDa membrane and afterwards diluted to appropriate concentrations for the binding assay (0.02–40 nM) with HBHA buffer using the formula 6 $OD_{405}$/min corresponds to 10 nM CekX–AP fusion protein. Cos cells were transfected with RAGS and ELF-1 cDNA respectively in 145 mm dishes and 36 h after transfection split into 35 mm six-well clusters. Binding assays were performed in triplicate for each concentration point, using the procedure of Cheng and Flanagan (1994). In brief, confluent Cos cells expressing one of the ligands were incubated with CekX–AP supernatant for 75–90 min and then washed four to five times with HBHA at room temperature. Cells were lysed in 400 µl 1% Triton X-100, 10 mM Tris pH 8.0, per well, centrifuged, treated at 65°C for 10 min and then assayed as 200 µl samples in a 96-well Dynatech ELISA reader, diluted 1:1 with 2× AP buffer (2 M diethanolamine pH 9.5, 2 mM $MgCl_2$, 5 mg *p*-nitrophenylphosphate/1500 µl). AP activity was calculated from the initial velocity of the reaction and data were transformed into molar concentrations for Scatchard analysis (Scatchard, 1949).

### *Quantitative binding of Cek4–AP fusion protein to sucrose membrane preparations*

To obtain a relative quantification of ELF-1 or RAGS present on Cos cell sucrose membrane preparations, a Cek4–AP binding assay was developed. Aliquots of membranes (50 µg) were pelleted (Eppendorf centrifuge 5403; 23 100 *g* for 8 min at 4°C), washed in 100 µl HBHA buffer, re-pelleted and resuspended in 100 µl Cek4–AP solution with an AP activity of 15 $OD_{405}$/min. Membranes were incubated on a rotary shaker (500 r.p.m.) at room temperature for 90 min, pelleted and washed twice in ice-cold HBHA to remove unbound Cek4–AP. The centrifugation time after these HBHA washing steps was reduced to 5 min. The membranes were resuspended in 100 µl 1% Triton X-100, 10 mM Tris–HCl pH 8.0, heated to 65°C for 10 min and re-pelleted (23 100 *g* for 8 min, 4°C). 50 µl of the supernatant was assayed as described in the previous paragraph. The determined AP activity ($mOD_{405}$/min/µg sucrose membranes) calculated from the initial velocity was taken as a relative value for ligand concentration. With this method, it is possible to compare membrane preparations of cells transfected with the same ligand. Comparison of ELF-1- with RAGS-transfected membranes should be considered with caution, due to different dissociation constants and unknown binding kinetics.

### *Collapse assay*

The procedure used in the collapse assay was essentially the same as that described by Cox *et al.* (1990). One nasal and one temporal retinal explant were grown overnight on a poly-D-lysine/laminin-coated glass coverslip in F12 culture medium. Aliquots of sucrose membrane preparations of Cos cells (protein concentrations were determined according to Walter *et al.*, 1987) were pelleted (23 100 *g* for 8 min, 4°C) and resuspended in F12 culture medium. After sonication on ice (twice for 15 s at 30 W, Branson sonicator) the working concentration was adjusted and 200 µl of membrane suspension was carefully applied to the retinal explants. Axonal growth cones were analysed using a charge-coupled device (CCD) camera. By using a computer-controlled scanning stage (J.Löschinger, unpublished data), 15 growth cones (eight of the temporal and seven of the nasal explant) could be observed simultaneously in a single experiment by time-lapse. Pictures were taken under manual control every 2–5 min, starting ~15 min before and ending 30 min after application of membrane vesicles. Pictures were digitized and stored on a computer hard disk. For analysis, the complete sequence was reloaded using the NIH Image 1.55 program.

### *Stripe assay*

The stripe assay experiments followed the protocol of Walter *et al.* (1987) with the following modification: before preparation of the membrane stripes, nucleopore filters were incubated in 20 µg/ml laminin in Hanks' medium for 2–3 h at 37°C. Afterwards, filters were washed in Hanks' medium and stored in the same medium until use. In stripe assay experiments, in which mock-transfected Cos membranes were tested against various dilutions of RAGS-containing membranes, both membrane types were diluted using untransfected Cos cells.

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