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In Vitro Guidance of Retinal Ganglion Cell Axons by RAGS, a 25 kDa Tectal Protein Related to Ligands for Eph Receptor Tyrosine Kinases

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Summary

The results of previous in vitro experiments indicate that a glycosylphosphatidylinositol (GPI)-anchored protein may play an important role in the guidance of temporal retinal axons during the formation of the topographically ordered retinotectal projection. We have purified and cloned a GPI-anchored, 25 kDa glycoprotein that is a good candidate for a molecule involved in this process. During the time of innervation by retinal ganglion cells, this protein is gradedly expressed in the posterior part of the developing tectum. In two different in vitro assay systems, the recombinant protein induces growth cone collapse and repulsion of retinal ganglion cell axons. These phenomena are observed for axons of temporal as well as nasal origin, indicating that an additional activity may be necessary to confer the nasotemporal specificity observed in previous assays. We named the protein RAGS (for repulsive axon guidance signal). The sequence of RAGS shows significant homology to recently identified ligands for receptor tyrosine kinases of the Eph subfamily.

Introduction

The processes in the developing brain by which specific groups of neurons become connected to each other can be divided into three major steps: pathway selection, target selection, and address selection (Goodman and Shatz, 1993). First, axons grow toward their target tissue with a leading growth cone at their tips, often traveling over long distances to find their correct target area. Growth cones encounter a variety of guiding influences, attractive or repellent, which can be due to either diffusible or substrate-bound molecules. Only recently, molecules with such functions have been identified and cloned (Luo et al., 1993; Serafini et al., 1994; Kennedy et al., 1994). In the second stage, after having reached their target area, axonal growth cones navigate within the target and set up a crude map of projections. In the final stage, axon terminals are remodeled within the target by retraction and expansion of processes, which leads to a highly tuned pattern of projections. This process involves synaptic competition and selective cell death (Goodman and Shatz, 1993; Udin and Fawcett, 1988).

A well-studied example of the formation of specific neuronal connections is the retinotectal projection, in which nasal axons of retinal ganglion neurons project to posterior tectum and temporal, dorsal, and ventral retinae are connected to anterior, ventral, and dorsal tecta, respectively (e.g., Holt and Harris, 1993). The collapse assay (Cox et al., 1990; Raper and Kapfhammer, 1990) and the stripe assay (Walter et al., 1987a) represent in vitro systems well suited to the study of mechanisms and molecules involved in axon guidance. In the collapse assay, membranes from anterior or posterior tectum are added to growing nasal or temporal retinal axons. Posterior membranes and, to a lesser extent, anterior membranes cause temporal growth cones to collapse. Nasal growth cones respond to posterior membranes with only a transient collapse (V. Happe and F. B., unpublished data).

In the stripe assay, temporal and nasal retinal axons grow on a substratum consisting of alternating lanes of anterior and posterior tectal membranes. At the boundaries between anterior and posterior material where the axons can simultaneously contact two different substrates, the axons are guided to grow onto the lanes consisting of membranes derived from their target area (Walter et al., 1987a; von Boxberg et al., 1993). Temporal axons, for example, grow on anterior lanes because they are kept from invading posterior lanes; this type of repulsion is caused by molecules that are bound to the membranes by a glycosylphosphatidylinositol (GPI) anchor. Guidance by repulsion seems to be related to the phenomenon of growth cone collapse (Walter et al., 1990b; Fan and Raper, 1995).

These observations were the starting point of our investigation. The spatiotemporal expression pattern of GPI-anchored membrane proteins in the tectum during the time of innervation by ganglion cell axons was systematically analyzed. Within the type of membrane preparation chosen, one candidate molecule was identified. The sequence of this 25 kDa glycoprotein, which includes features typical of GPI-anchored proteins, and its expression pattern in the developing tectum are consistent with its playing a significant role in axon guidance. In the collapse assay, membranes from COS cells transfected with the corresponding cDNA cause growth cone collapse of temporal, but also of nasal ganglion cell axons. In the stripe assay, both types of retinal axon are repelled from stripes containing the recombinant protein. We named the 25 kDa protein that causes collapse and repulsion RAGS (for repulsive axon guidance signal).

Results

Analysis of the Expression Pattern of GPI-Anchored Proteins in the Chicken Tectum During the Time of Innervation by Retinal Ganglion Cell Axons

In the stripe assay, retinal ganglion cell axons are offered the choice of growing on either anterior or posterior tectal

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membranes (Walter et al., 1987a). Temporal axons are repelled by posterior membranes and grow on anterior stripes (Walter et al., 1987b). After incubation of the tectal membranes with phosphatidylinositol-specific phospholipase C (PI-PLC), a bacterial enzyme that specifically cleaves GPI-anchored proteins from the membrane surface (Low, 1989), the posterior tectal membranes lose their repellent activity for temporal axons, which then cross freely over the striped carpet with no obvious preference for either of the membrane types (Walter et al., 1990b). The posterior membrane repellent activity is already expressed by the time retinal axons arrive at the tectum (embryonic day 6 [E6]) and lasts up to about E12, when the majority of axons have reached their target area (Walter et al., 1987a). Therefore, three criteria assumed for a candidate guidance molecule for temporal axons are GPI anchorage, expression from E6–E12, and higher expression in posterior than in anterior tectum. For our approach, we relied on data showing that only a few proteins within a given cell type are GPI anchored (Rosen et al., 1992).

Using two-dimensional (2D) gel electrophoresis, which provides much more information and a higher resolution than conventional 1D gels, proteins were separated on the basis of weight and electric charge (O'Farrell, 1975). Anterior and posterior thirds of chicken tecta of embryonic days 7, 9, and 13 were isolated. Membrane extraction by urea treatment was followed by incubation at pH 11.5 to remove peripherally bound membrane proteins. After incubation with the PI-PLC, membranes were pelleted by centrifugation. The supernatant, which contained proteins originally GPI anchored and now released from the membranes, was analyzed by 2D gel electrophoresis (Figures 1 and 2). This procedure yielded a final enrichment factor of about 1000 for GPI-anchored proteins. The appearance of F11 in the supernatant (see Figure 1, marked by a triangle) served as an internal control for the success of the PI-PLC treatment. F11 is a GPI-anchored cell adhesion molecule with an apparent molecular weight of 130 kDa (Brümmendorf et al., 1989).

A number of proteins were spontaneously released from membranes even during incubation without the enzyme, leading to considerable background (see Figure 1, bottom). As this background might be due to soluble proteins being trapped in membrane vesicles, the protein patterns of anterior and posterior tectal membranes were compared after incubation with or without PI-PLC to detect GPI-anchored proteins.

A careful inspection of numerous 2D gels led to the unambiguous identification of a protein with a molecular mass of 25 kDa (Figures 1 and 2, arrows). This 25 kDa protein is the only one found in this preparation that fulfills all three criteria mentioned above, i.e., differential spatial expression along the anterior–posterior axis, temporal expression limited to early developmental stages, and GPI anchorage to plasma membranes. We named this protein RAGS (see below).

As this protein represents an interesting candidate for a tectal axon guidance molecule, we decided to purify and clone it to unravel its function in the formation of the retinotectal projection.

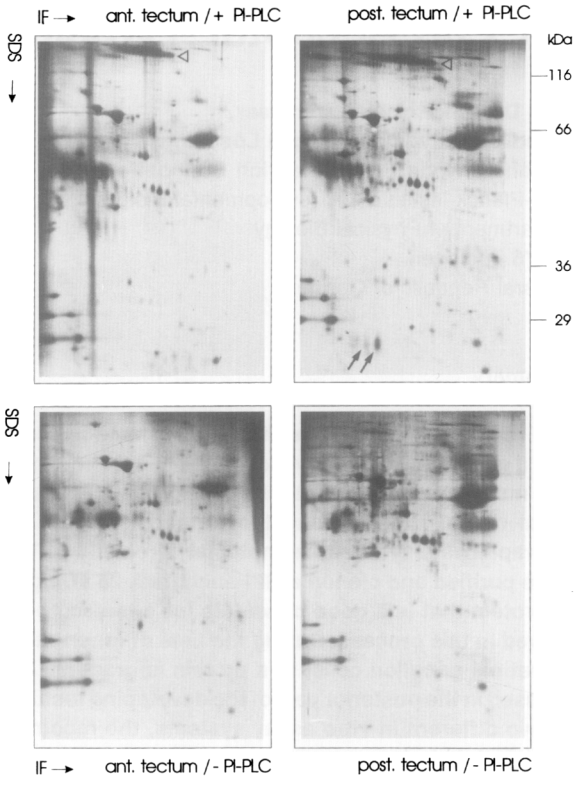

Figure 1. 2D Gel Analysis of E9 Chicken Tectal Proteins

Membranes from anterior and posterior thirds of tecta were incubated with or without (indicated by plus or minus, respectively) PI-PLC. Membranes were then pelleted, and supernatants were concentrated and subjected to electrophoresis. In the vertical dimension, proteins were separated according to molecular weight (SDS dimension); perpendicular to this, they were separated based on their electric charge (IF dimension); proteins migrate from the acidic area (left) into the basic area (right). Proteins were visualized by silver staining. Arrows indicate RAGS spots; the 130 kDa GPI-anchored F11 protein is marked by a triangle.

Purification, Cloning, and Sequence of RAGS

The isolation procedure of the RAGS protein spot is essentially a scale-up of the analytical method described in the previous section. About 2000 E9 and E10 embryos were needed to obtain sufficient amounts of material for peptide sequencing. This material was separated in a total of ten preparative 2D gels. After staining with Coomassie blue R250, the most prominent spot in the RAGS series was cut out from the gel. In this way, 6 μg of pure protein was obtained. Assuming that proportional to other proteins no significant amounts of RAGS were lost during purification, the protein was found to comprise less than 1/10,000 of total posterior tectal membrane proteins.

The isolated protein was digested with the protease LysC and eluted from the gels. The resulting peptides were separated by high pressure liquid chromatography (HPLC), and NH_2-terminal sequences of selected peptides were determined. Two of these are shown in Figure 3A. From one of these sequences, a degenerate oligonucleotide was designed, which was then used to screen a chicken cDNA library made from E8 posterior tectum (see Experimental Procedures). Some of the cDNA clones isolated

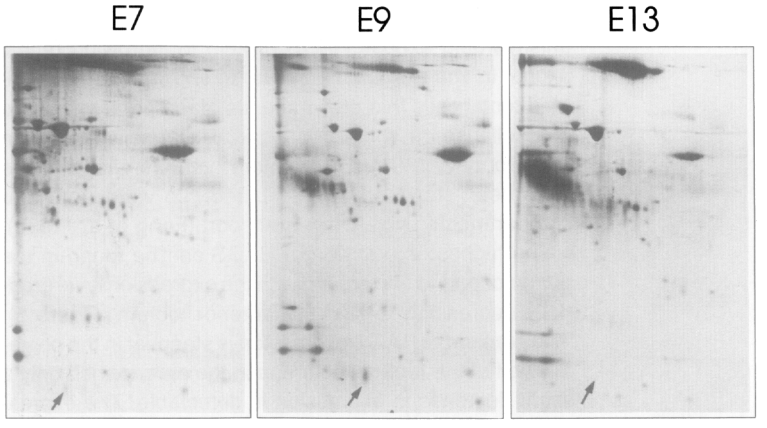

Figure 2. 2D Gel Analysis of Chicken Tectal Proteins from Various Developmental Times

Fractionated membrane proteins from posterior thirds of tecta from E7, E9, and E13 were separated by 2D gel analysis (see Figure 1). Arrows point to RAGS spots (E7 and E9) and to the area where RAGS spots are expected at E13. In experiments in which significantly more protein was loaded (3-fold), no RAGS spots were detected at E13 (data not shown).

were sequenced, and one sequence is shown in Figure 3A. The same sequence was found in other independently isolated cDNA clones.

The sequence obtained contains only one long open reading frame (ORF). At the extreme NH_2-terminus, either of two closely spaced methionine codons in-frame might be used for initiation of translation. The first methionine presumably will be used, as sequences surrounding this ATG fit well with rules proposed by Kozak (1989) for initiation of translation. The deduced amino acid sequence includes both the peptide from which the oligonucleotide was designed to screen the cDNA library and the other peptide obtained from protein sequencing.

Sequence analysis of the ORF shows regions of high hydrophobicity at the NH_2- and COOH-termini (Figure 3B). The NH_2-terminal stretch of 20 amino acids comprises a putative signal sequence (von Heijne, 1985), which would direct the protein to the secretory pathway. The hydrophobic COOH-terminal region is typical of GPI-anchored proteins (Ferguson and Williams, 1988).

The predicted molecular mass of the mature protein (after cleavage of signal peptide and COOH-terminal domain) is 21.1 kDa. This correlates well with the molecular mass of the isolated protein if the effect of glycosylation on electrophoretic mobility is taken into account. The isoelectric point of the amino acid sequence is about 6.0, consistent with the focusing of the RAGS spots in the acidic part of 2D gels. In the NH_2-terminal region, a consensus sequence for N-linked glycosylation is predicted.

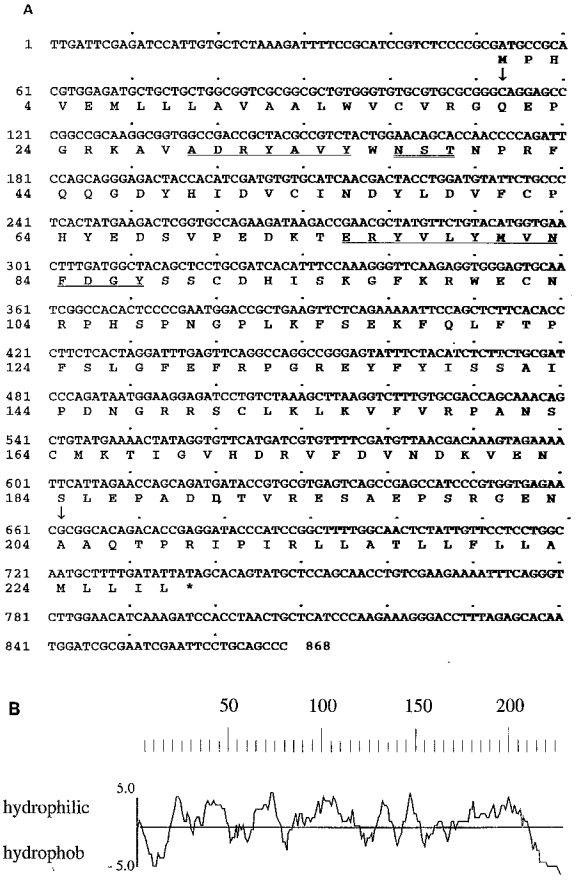

Figure 3. The Nucleotide and the Predicted Amino Acid Sequence of RAGS

(A) The positions of nucleotide and amino acid residues are given to the left. Two peptide sequences obtained by microsequencing are underlined. A potential N-linked glycosylation site is doubly underlined. Putative cleavage sites of the NH_2-terminal signal peptide and the COOH-terminal hydrophobic peptide are indicated by arrows.

(B) Kyte–Doolittle hydrophobicity plot of the predicted RAGS amino acid sequence. This analysis was done using GCG software (Genetics Computer Group, 1992).

Northern Blot Analysis of RAGS Expression during Development

A 310 bp HindIII fragment covering the 3′ portion of the cDNA was used as a probe to analyze the expression pattern of the RAGS molecule on the transcriptional level. One prominent transcript of about 6 kb is clearly detected with a distinct difference in the expression level between anterior and posterior parts of the tectum. During the developmental period analyzed, RNA expression of RAGS is highest at E7 and decreases to a significantly lower level by E13 (Figure 4). The spatially invariant expression pattern throughout development is a strong indication that RAGS transcription in the tectum is controlled in a region-

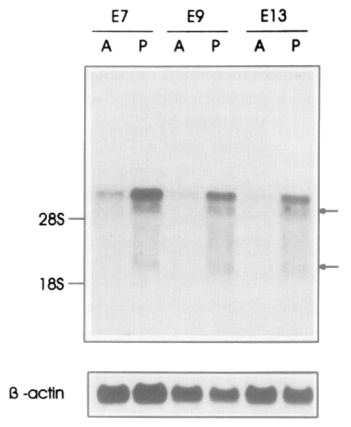

Figure 4. Northern Blot Analysis of RAGS Expression in the Developing Chicken Tectum

Poly(A)$^+$ RNA (5 μg per lane; Sambrook et al., 1989) from anterior and posterior thirds of tecta of the indicated developmental age was loaded. Under high stringency conditions (final washes of the filters in 0.1 × SSC, 0.1% SDS at 65°C), the RAGS probe detects a major transcript of about 6 kb and minor transcripts of 5.0 kb and 2.5 kb (indicated by small arrows). Positions of size markers are indicated to the left. The blot was rehybridized with a chicken β-actin probe to monitor RNA content in each lane.

specific manner and does not reflect a maturational gradient in the tectum.

Additional transcripts with a lower expression level of about 5 and 2.5 kb can be detected in the Northern blot (Figure 4) and follow the expression pattern of the main 6 kb transcript. It is possible that these transcripts encode splicing variants of the GPI-anchored RAGS molecule. Alternatively, they might represent highly related sequences derived from related genes.

The discrepancy between the appearance of a RAGS transcript at E13 and the failure to detect a corresponding GPI-anchored protein at the same time in silver-stained 2D gels might be due to different detection sensitivities of the two methods. The same might hold true for the anterior/posterior distribution, where RNA, but not protein, is seen in anterior tectum. An interesting alternative is that a posttranslational control mechanism is acting on top of the transcriptional control.

In Situ Hybridization Analysis of RAGS Expression

In situ hybridization analysis was performed to determine the exact distribution of RAGS mRNA in tissue sections of the tectum, which could not be deduced from the Northern blots. The strongest expression of RAGS is detected in the deeper cell layers that line the mesencephalic ventricle (Figure 5). As retinal axons invade the tectum in superficial layers close to the pia mater, the localization of RAGS mRNA to the innermost cell layers may seem surprising at a first glance. However, the cell layers that express RAGS mRNA contain radial glia cells, which span the distance to the superficial layers and which possess so-called end feet at the tectal surface. These end feet are in close contact with the traveling retinal axons. We hypothesize that RAGS protein might be transported into superficial layers and into these end feet (see Discussion). This mechanism would expose RAGS protein to ingrowing retinal axons. In line with this, it has been shown using dissociated tectal cells that growth cones of temporal retinal axons collapse on contact with radial glial cells (Johnston and Gooday, 1991). Furthermore, various glial proteins have been shown to be distributed into various cellular compartments by selective transport (Feng et al., 1994).

The highest expression of RAGS can be found in the posterior part of the tectum, with the expression level gradually decreasing toward the anterior tectum (Figure 5). The gradient of RAGS expression is steepest in the posterior half of the tectum, whereas in the anterior half only a slight decrease in expression is detectable. The expression pattern fits well with data obtained in experiments carried out by Bonhoeffer and Huf (1982) and by Walter et al. (1987b). In the latter study, membranes from tecta, which had been cut into four parts perpendicular to the anterior–posterior axis, were analyzed in pairs in the stripe assay. It was found that membranes from more posterior tectal quarters exert stronger repellent activity on temporal axons than membranes from more anterior quarters. This was true for all combinations except for the two most anterior quarters, in which a clear decision of temporal axons could not be observed. This was taken as an indication that the gradient was weak or absent in the anterior tectum.

A weak expression of RAGS mRNA can also be detected in a more superficial layer, the stratum griseum centrale (SGC). As is true for the strong expression in the deeper cell layers, the expression in the SGC is high in the posterior tectum and gradedly decreases toward the anterior pole (Figure 5).

RAGS transcripts can also be found within the tegmental hemisphere located ventral to the tectum and in preparations containing both hindbrain and spinal cord structures,

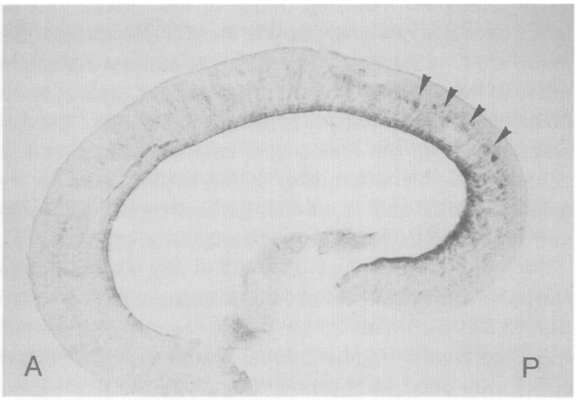

Figure 5. In Situ Hybridization Analysis of RAGS mRNA Expression in the Developing Tectum

Cryostat sections of E9 tecta were hybridized with digoxigenin-11-UTP antisense RAGS cRNA. The expression pattern in a sagittal section after an overnight color reaction is shown. Anterior (A) and posterior (P) poles of the tectum are indicated. Hybridization within the stratum griseum centrale (SGC) is indicated by arrow heads. Hybridization of the corresponding sense strand gave essentially no staining (data not shown).

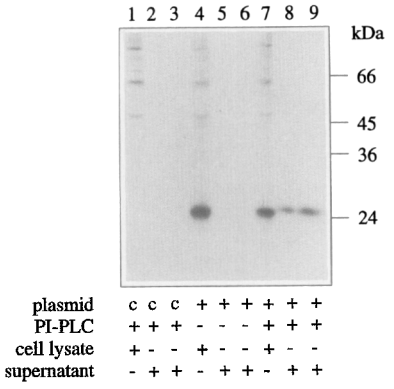

Figure 6. PI-PLC Sensitivity of Recombinant RAGS

Membranes from RAGS (plus) and mock-transfected (c) COS cells were incubated with or without PI-PLC. After ultracentrifugation, membrane pellets and supernatants were immunoprecipitated using a RAGS-specific polyclonal antiserum (see Experimental Procedures). Lanes 2, 5, and 8 show immunoprecipitations of native; lanes 3, 6, and 9 show immunoprecipitations of denatured membrane proteins.

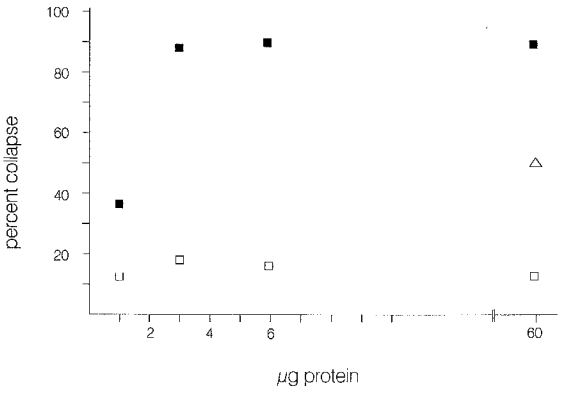

Figure 7. Quantification of the Growth Cone Collapse–Inducing Activity of RAGS-Transfected COS Cell Membranes

Membranes from different sources were analyzed for their collapse-inducing activity on retinal (nasal and temporal) growth cones. Closed boxes, RAGS-transfected COS cell membranes; open boxes, mock-transfected COS cell membranes; open triangles, E9 posterior tectal membranes. Growth cone collapses observed after prolonged incubation with membranes (>30 min) have not been taken into account.

but not in preparations of forebrain (data not shown). The expression of the RAGS protein is not strictly tectum specific, and it may function in other parts of the brain.

Analysis of the Biological Function of Recombinant RAGS

RAGS was further analyzed in two different in vitro bioassay systems to test for a possible involvement of this protein in axonal guidance processes during formation of the retinotectal projection.

For this purpose, the cDNA was cloned into the expression vector pCDM8 (see Experimental Procedures), in which the expression of RAGS is controlled by the strong cytomegalovirus (CMV) promoter. This plasmid was introduced into COS cells using the calcium phosphate technique (Chen and Okayama, 1987). After ^{35}S labeling, cell lysates were prepared and biochemically analyzed for the expression of RAGS protein. By using an antiserum raised against bacterially expressed RAGS (see Experimental Procedures), a protein of 25 kDa could be specifically immunoprecipitated from transfected COS cells (Figure 6). After PI-PLC treatment of these cell lysates, RAGS could be detected also in the supernatant, indicating that a significant amount of this protein is attached to COS cell membranes by a GPI anchor. The remaining RAGS, which could not be released from COS cell membranes by PI-PLC treatment (Figure 6), might represent incompletely processed, peptide-anchored RAGS molecules. Owing to the high expression of RAGS in COS cells, those factors that are required for processing of the immature protein into a GPI-anchored protein might be saturated.

In the collapse assay (Cox et al., 1990; Raper and Kapfhammer, 1990), retinal ganglion cell axons growing on a laminin-coated surface are exposed to membrane fragments of interest by pipetting these directly into the medium containing the retinal explants. The behavior of a large number of growth cones before and after contact with the sedimenting membrane fragments was followed simultaneously in a time-lapse analysis using a combination of a computer-controlled scanning stage and a charge-coupled device (CCD) camera. In this experiment, membranes from RAGS-transfected COS cells specifically induced growth cone collapse of temporal axons in a concentration-dependent manner (Figure 7). Strikingly, nasal growth cones also showed a similar response to RAGS-containing COS cell membranes. To exclude the possibility of a nonspecific collapse-inducing activity of the COS cell membranes themselves, control experiments were performed with mock-transfected COS cells. When exposed to these membranes, the overwhelming majority of both nasal and temporal growth cones was not affected at all. A rough estimation of the biological activity of RAGS COS membranes can be obtained by comparison with posterior tectal membranes. Whereas about 60 μg of posterior tectal membranes are required to induce 50% collapse of temporal growth cones (Müller, 1992), about 1 μg of RAGS COS cell membranes is sufficient to obtain the same effect on either temporal or nasal axons (Figure 7).

An example of a time-lapse analysis of the interaction between the growth cone of a temporal retinal axon and membrane particles derived from RAGS-transfected COS cells is illustrated in Figure 8. A migrating temporal growth cone (Figures 8A and 8B) comes into contact with sedimenting particles (Figure 8C), is paralyzed for a brief time (Figure 8D), and within a few minutes, the whole growth cone structure collapses and the axon is retracted (Figures 8E and 8F). Figures 8G–8I demonstrate that even very high amounts of mock-transfected COS cell membranes do not affect the migration of temporal growth cones.

Usually, RAGS-induced collapse of retinal growth cones was observed 3–15 min after application of the probe; however, a fraction of temporal axons (about 15%) were resistant to RAGS COS cell membranes. Interestingly, this number is identical for studies using posterior tectal membranes (Cox et al., 1990). The biological significance of this

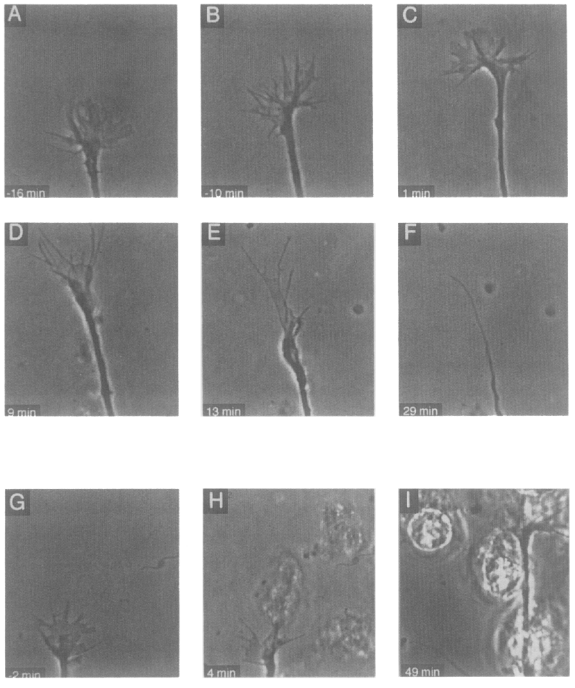

Figure 8. Time-Lapse Analysis of a RAGS-Evoked Growth Cone Collapse

A series of video images showing the behavior of a temporal retinal growth cone before (A and B) and after (C–F) addition of membrane particles derived from RAGS-transfected COS cells. Times at which individual frames were taken are given in minutes at the bottom left of each picture. Negative numbers indicate time intervals before application of the probe. (G)–(K) illustrate the migration of a temporal axon in high amounts of membranes derived from mock-transfected cells.

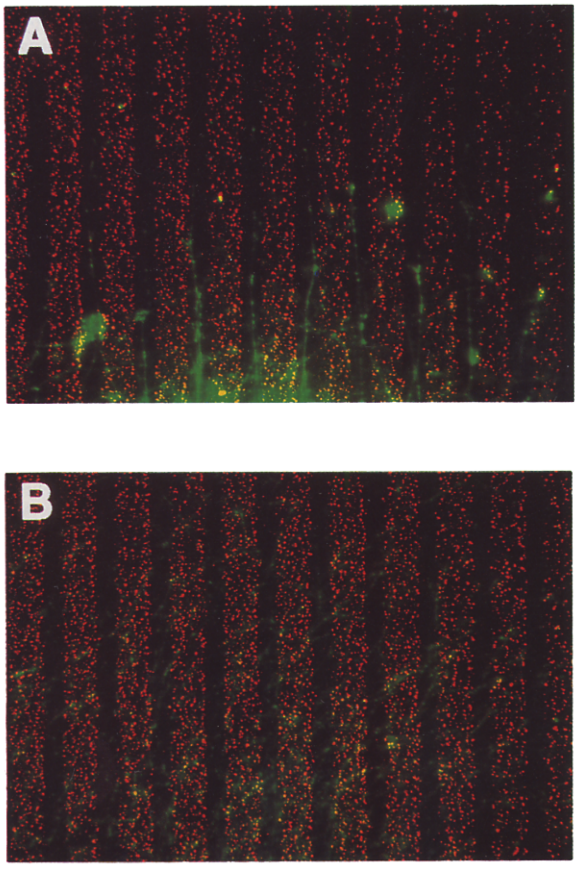

Figure 9. Guidance of Retinal Axons by RAGS in the Stripe Assay

(A) Nasal (left half) and temporal (right half) axons were given the choice of growing on membranes derived from either RAGS-transfected COS cells (labeled with rhodamine–isothiocyanate [RITC] fluorescent beads) or from mock-transfected COS cells.

(B) Outgrowth of retinal axons on solely mock-transfected COS cell membrane stripes. Stripes prepared first are labeled with RITC-fluorescent beads. Retinal ganglion cells were stained with DiAsp during preparation of retinal tissue. Membrane stripes were prepared on laminin-coated filters to provide an outgrowth-promoting substrate.

phenomenon is unclear at present; however, it indicates a heterogeneity within the population of temporal axons, possibly reflecting the composition of receptors expressed on their growth cones.

To extend the functional analysis, the stripe assay system was used to test the ability of recombinant RAGS to guide retinal axons in vitro. Alternating stripes derived from RAGS COS cell membranes and from membranes derived from mock-transfected cells were prepared on laminin-coated filters. Laminin was used to provide an axonal outgrowth–promoting substrate. An analysis of retinal explants growing on these stripes showed a strong deflection of nasal and temporal retinal axons from membrane stripes containing RAGS, i.e., retinal axons almost exclusively grew on membranes derived from mock-transfected COS cells (Figure 9A). Normal outgrowth of retinal axons was observed in control experiments in which striped carpets were prepared containing only COS cell membranes (Figure 9B).

The property of RAGS to deflect both temporal and nasal axons is apparently different from the activity of posterior tectal membranes, which do not repel nasal axons in the stripe assay. However, in the collapse assay, nasal axons respond to posterior membranes with a growth cone collapse, albeit transient, indicating that posterior tectal membranes also possess a low collapse-inducing activity for nasal axons. Therefore, the possibility remains that nasal axons are repelled in these stripe assay experiments by unphysiologically high concentrations of RAGS due to the strong expression in COS cells. It would then be expected that lower concentrations of RAGS would guide temporal, but not nasal axons. To test this possibility, a series of stripe assay experiments was performed in which mock-transfected COS cell membranes were tested against various dilutions of RAGS-containing cell membranes. In none of these experiments could a concentration of RAGS be found in which nasal and temporal axons showed a differential response. At a 3-fold dilution, the growth preference of nasal and temporal axons was significantly reduced; at a 10-fold dilution, neither nasal nor temporal axons showed a preference for any of the membranes.

It can thus be concluded that the RAGS molecule possesses an activity that guides retinal axons in vitro by repulsion of their growth cones but does not seem to discriminate between nasal and temporal axons.

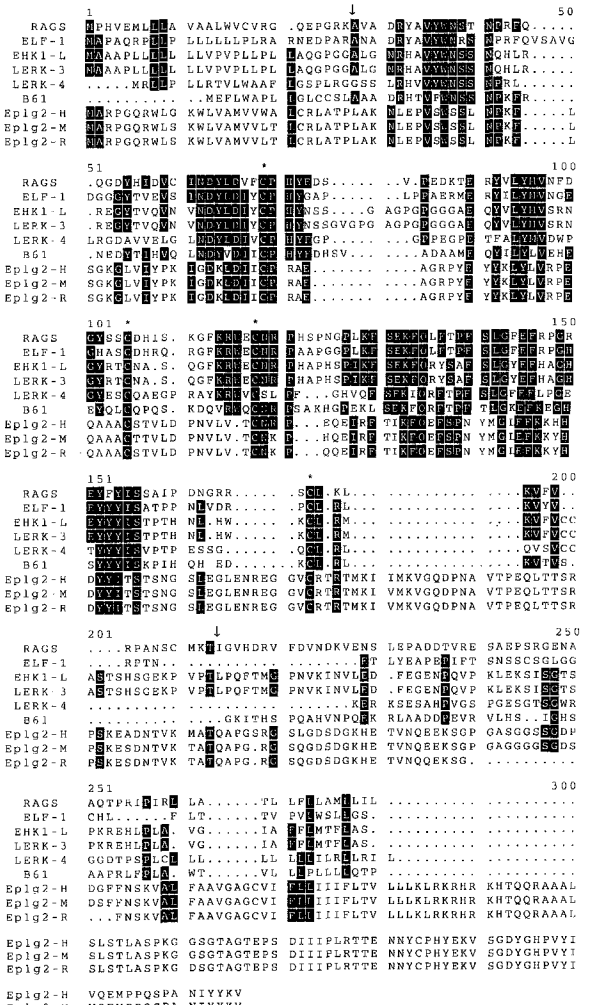

Figure 10. Alignment of Amino Acid Sequences of Eph Ligand Family Members

Amino acids identical in at least five out of the nine proteins are shown in inverse type. The COOH-terminal thirds of these proteins, which include the membrane anchorage signals, do not show significant similarities. The four cysteine residues common to all nine proteins are marked by asterisks. Limits of the arbitrarily set core region are indicated by arrows. EHK1-L and LERK-3 as well as Eplg2-H (referred to as LERK-2 in the main text), Eplg2-M, and Eplg2-R represent species homologs. An insertion of four amino acids in LERK-3 compared with EHK1-L is noted.

RAGS is Homologous to Ligands of Tyrosine Kinase Receptors of the Eph Subtype

A search in DNA and protein data bases for sequence similarity to RAGS showed significant homology to proteins recently identified as ligands for receptor tyrosine kinases (RTKs) of the Eph subtype (Tuzi and Gullick, 1994), which were originally named B61 (Holzman et al., 1990; Bartley et al., 1994; also known as LERK-1 [Beckmann et al., 1994; Davis et al., 1994]), LERK-2 (Beckmann et al., 1994; Davis et al., 1994; also known as Eplg-2 [Fletcher et al., 1994a, 1994b] and Cek5 ligand [Shao et al., 1994]), ELF-1 (Cheng and Flanagan, 1994), EHK1-L (Davis et al., 1994; also known as LERK-3 [Kozlosky et al., 1995]) and LERK-4 (Kozlosky et al., 1995).

The Eph subclass represents the largest known subfamily of RTKs, of which seven members have been identified in chicken (Pasquale, 1991; Sajjadi et al., 1991; Sajjadi and Pasquale, 1993; Siever and Verderame, 1994). Other subfamilies of RTKs include the neurotrophin, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor receptors (van der Geer et al., 1994). Although it is known that other RTK subfamilies play a key role during development and differentiation, a specific function of Eph subtype receptors during neuronal development has not been described up to now.

The first RTK ligand characterized, B61, was originally isolated in a screen of a human endothelial cell line for genes induced by tumor necrosis factor α (Holzman et al., 1990). The authors could not attribute a function to this gene. Bartley and coworkers reidentified B61 in a screen for cell culture supernatants containing receptor-binding

Table 1. Homology between Individual Members of the Eph Ligand Family

	ELF-1	EHK1-L	LERK-3	LERK-4	B61	Eplg2-H	Eplg2-M	Eplg2-R
RAGS	53.2	41.1	41.1	42.0	45.5	25.8	29.0	26.6
	(68.4)	(52.5)	(52.5)	(49.2)	(53.8)	(28.9)	(28.9)	(28.1)
ELF-1		47.8	47.8	46.0	44.1	29.1	28.4	30.5
		(58.5)	(58.5)	(53.1)	(53.4)	(32.8)	(32.1)	(32.1)
EHK1-L			99.6	43.5	41.6	29.6	28.8	25.1
			(99.3)	(52.7)	(51.5)	(31.7)	(31.7)	(29.1)
LERK-3				43.5	43.1	30.0	29.3	25.6
				(52.7)	(53.8)	(32.4)	(32.4)	(30.0)
LERK-4					38.5	32.8	32.6	31.6
					(43.1)	(30.4)	(30.4)	(30.4)
B61						33.2	29.3	29.9
						(36.5)	(36.5)	(35.7)
Eplg2-H							95.7	96.4
							(95.8)	(96.4)
Eplg2-M								98.8
								(98.8)

The complete coding region and the central more strongly conserved part of these proteins including all four conserved cysteine residues (indicated by arrows in Figure 10) have been considered. Values indicate percent identity; values in brackets refer to the core region.

activity and showed it to bind to an immobilized eck RTK (Lindberg and Hunter, 1990). LERK-2, ELF-1, EHK1-L (LERK-3), and LERK-4 were identified in expression screens in which cells transfected with pools of cDNAs were screened with the extracellular domain of either the elk RTK (Letwin et al., 1988), the Mek4/Sek RTK (Gilardi-Hebenstreit et al., 1992; Nieto et al., 1992), the EHK1 RTK (Maisonpierre et al., 1993), or the hek RTK (Wicks et al., 1992).

Based on the sequence similarities between the molecules outlined in Figure 10 and Table 1, it is most likely that RAGS also is a member of the Eph ligand family.

Interestingly, of the ligand molecules identified so far, mouse ELF-1, like chicken RAGS, is expressed in the developing mesencephalon. On the basis of a sequence homology of about 65% between these two proteins, it is unlikely that RAGS is the chicken homolog of ELF-1, since the species homologs of ligands and receptors within this family apparently show a very high degree of amino acid sequence homology (>90% identity [see Table 1; Fletcher et al.,1994b]). However, the only way to prove the relationship between chicken RAGS and mouse ELF-1 will be to clone either the chicken homolog of mouse ELF-1 or, vice versa, the mouse homolog of chicken RAGS.

Discussion

Inhibitory and repellent molecules that specifically restrict axonal growth are believed to contribute to the formation of ordered projections in the central nervous system (e.g., Walter et al., 1987a; Fawcett et al., 1989; Bandtlow et al., 1990; Davies et al., 1990; Raper and Grunewald, 1990; Moorman and Hume, 1990; Pini, 1993; for review see Goodman and Shatz, 1993). Derived from findings of in vitro studies, a GPI-linked molecule has been proposed to play an important role in guiding temporal retinal axons to their correct topographic position in the optic tectum by repelling this class of growth cones from the posterior part of the tectum. We have taken a descriptive, nonfunctional approach toward cloning GPI-linked molecules conferring this activity. This nonfunctional method was necessary, because the repellent activity could be solubilized only in the presence of high concentrations of urea. This, however, diminished the chances of obtaining good separation with chromatographic methods as proteins tend to smear in high concentrations of urea. The reason for the poor solubility of this activity is presumably the association of GPI-linked proteins with a special lipid environment (Brown and Rose, 1992), from which they are difficult to purify. Attempts to characterize the repellent activity from supernatants of cell membranes after PI-PLC treatment were unsuccessful, probably because clustering mediated by the GPI anchor is essential for the expression of these activities (cf. Davis et al., 1994).

We decided to search for molecules that are GPI anchored, show a high expression in posterior and a low expression in anterior tectal membranes, and are expressed during the time of innervation by retinal axons (E6–E12). For the analysis, we used high resolution 2D gel electrophoresis (O'Farrell, 1975) and found one protein with an apparent molecular weight of 25 kDa showing the expression pattern expected for the repellent molecule. However, we can not exclude that additional undetected proteins show a similar expression pattern as this molecule.

Both the deduced amino acid sequence of the RAGS cDNA and the PI-PLC treatment used for the isolation support the belief that we have cloned a GPI-anchored protein. The RNA expression data are compatible with the protein expression analysis, showing that RAGS is differentially expressed in the tectum during the time of ingrowth of retinal axons (E6–E12). The transcript level is significantly down-regulated after completion of this process at around E12. Nothing is known about possible functions later in development (from E13 on), e.g., in positioning of collateral branches (Simon and O'Leary, 1992; Roskies and O'Leary, 1994).

An investigation of the spatial expression pattern in the tectum using in situ hybridization revealed that RAGS is expressed in a continuous gradient, with a high expression in the most posterior part and a gradual decrease toward the anterior pole. This graded expression in particular fits very well with the expected expression pattern of a repellent molecule for temporal axons, as these axons might be steered away from the posterior tectum by interaction with this factor. It was shown that in vitro temporal retinal growth cones can be guided by gradients of surface-associated molecules from the posterior tectum (Baier and Bonhoeffer, 1992). In fact, it appears that growth cones detect gradients better than steps. This graded expression pattern is also in agreement with a theoretical model proposed by Gierer (Bonhoeffer and Gierer, 1984; Gierer, 1987) to explain the development of the retinotectal projection.

In situ hybridization analysis demonstrated that the RAGS mRNA can for the most part be detected in the inner cell layers of the tectum. However, as ingrowing axons navigate in superficial layers of the tectum, the expression of RAGS in deeper layers raises the question how RAGS could then affect these axons. These deeper layers contain radial glial cells, which project their processes well into superficial layers; especially, glial end feet have been shown to be in close contact with ingrowing retinal axons. It has been demonstrated by Feng et al. (1994) that GFAP and BLBP transcripts are confined to nuclei of radial glia cells, but that the corresponding proteins can also be found in multiple superficial layers. In case of GPI-anchored proteins, it has been postulated that the GPI anchor itself is functionally involved in the transport of the attached protein moiety into certain compartments of a cell (e.g., in the polarized transport of GPI-anchored proteins in epithelial cells [Lisanti et al., 1988; Brown and Rose, 1992]). It would then be tempting to speculate that the GPI anchor serves to transport the protein from the cell body to the glial end feet on the tectal surface, exposing the RAGS molecule to the ingrowing retinal axons.

As a step toward elucidating the function of RAGS during formation of the retinotectal projection, the collapse assay demonstrated that membranes containing recombinant RAGS specifically induce collapse of growth cones of temporal and also nasal retinal axons. Collapsin, a molecule

recently purified and cloned from chicken brain (Luo et al., 1993) that specifically induces the collapse of dorsal root ganglion growth cones, has been shown to exert its function through net depolymerization of F-actin filaments (Fan et al., 1993). It will be interesting to analyze whether a similar mechanism is involved in RAGS-mediated retinal growth cone collapse.

In principle, the collapse assay cannot detect unequivocally a guidance function of RAGS. This is inherent in the assay itself. Here, growth cones are exposed instantly from all sides to membrane particles containing collapse-inducing activity. In vivo, the interaction between ingrowing retinal axons and tectal membranes is expected to be more subtle. Axonal growth cones are probably guided in the tectum by slightly increasing concentrations of repellent activity, which may be sensed only by a subregion of the growth cone. Additionally, habituation over time might be involved in the in vivo response (Walter et al., 1990a).

Similarly, the stripe assay does not involve a gradual increase of repellent activity, but a sharp delineation between different membrane types. However, it has the advantage that during axonal growth presumably only a restricted part of the growth cone comes into contact with stripes containing the repellent activity. Therefore, the stripe assay should be better suited than the collapse assay to uncover any guidance activity in vitro.

Our detection of a repellent activity of RAGS for retinal axons in the stripe assay confirms the idea that the phenomena of growth cone collapse and axonal guidance are closely related (Walter et al., 1990b). It appears that the modes of spatial and temporal action of molecules like RAGS are critical factors for growth cone collapse and axonal guidance. Interestingly, it was recently shown that collapsin, so far known to induce full growth cone collapse of dorsal root ganglion cell axons, can function in steering away these axons by inducing a localized growth cone collapse (Fan and Raper, 1995).

Although the spatiotemporal expression pattern and the functional activities of RAGS fit very well into a concept in which this molecule is involved in the guidance of temporal axons, the sensitivity of nasal retinal axons to RAGS in the collapse assay and in the stripe assay is at present not fully understood. It has been shown that posterior tectal membranes contain a weak activity for induction of a transient growth cone collapse of nasal axons (V. Happe and F. B., unpublished data). Therefore, high amounts of RAGS in membranes of transfected cells could be the cause of the deflection of nasal axons in the stripe assay. Lower concentrations of RAGS should then be ineffective for nasal axons while still effective for temporal axons. In contradiction to this assumption, stripe assays in which serial dilutions of RAGS COS membranes were tested against mock-transfected cell membranes did not reveal a concentration-dependent difference of RAGS action on nasal versus temporal axon behavior. The conclusion from these experiments is that RAGS on its own possesses a general guidance activity for retinal axons and, therefore, represents only part of the activity exerted by posterior tectal membranes on retinal axons.

Other molecules associated with RAGS may modulate the repellent activity. A candidate for a molecule that might interact with RAGS is a 33 kDa molecule described in a previous publication by Stahl et al. (1990). This protein was identified using a different purification scheme. The 33 kDa molecule is GPI anchored, expressed in a similar spatiotemporal expression pattern as RAGS, and was shown by Stahl et al. (1990) to be active in the collapse assay and to guide retinal axons in the stripe assay. The purification and cloning of the 33 kDa molecule is underway (B. Müller, personal communication). Within a particular step of the purification protocol used for the isolation of RAGS, i.e., the incubation of posterior tectal membranes in high pH to remove peripherally bound proteins, possibly the 33 kDa protein is unspecifically released from the membranes, explaining the absence of this protein from 2D gels shown in Figures 1 and 2.

Very recently, Winslow et al. (1995) reported the cloning of AL-1, which seems to represent the human homolog of chicken RAGS (91% amino acid identity). Using a soluble form, they demonstrated an involvement of this protein in axon bundle formation of cortical neurons. Further investigations will possibly show that axon bundling and axon guidance have a similar basis.

RAGS is a member of the recently identified family of ligands for the Eph RTKs. It is known that a number of different receptors of this subclass are expressed in the developing nervous system and also in the embryonic retina (e.g., Sajjadi and Pasquale, 1993; Henkemeyer et al., 1994; Pasquale et al., 1994). Their expression on axons of retinal ganglion cells (Pasquale et al., Soc. Neurosci. 1994, abstract) makes them possible candidates for mediating responses to guidance cues. These guidance cues then would be ligands for the Eph RTKs that are expressed along the axonal pathways (including the retina). Some of these ligands, like RAGS, might be expressed in overlapping domains in the target area, the optic tectum, thereby defining one of the potentially multiple networks that provide positional information that guide traveling retinal axons. This would mean that the guidance of retinal axons is controlled not by a single, but by a number of different interactions between ligands and their corresponding receptors. Each of these receptor/ligand pairs might, for example, influence the level of activation of certain second messenger systems in the growth cone; integration of all these different signals might finally determine the overall growth behavior of the axonal growth cone. A single component, e.g., a ligand, taken out of this network and tested individually in an in vitro assay might therefore exert a different effect on retinal growth cones than it does within a complex network expected for the in vivo situation.

In this context, it is conceivable that nasal axons, but not temporal axons, express a certain Eph RTK, which, after activation by the corresponding ligand, renders these axons insensitive to the repellent activity of RAGS. This postulated modulator could in principle be expressed in the entire tectum or more restrictedly in the posterior region in association with RAGS. Intriguingly, within the retinotectal projection, at least two Eph RTKs are differentially expressed along the anterior–posterior (U. D., unpublished data) and the dorsal–ventral axes (Pasquale et al.,

Soc. Neurosci. 1994, abstract) in patterns consistent with the idea that specific interactions between pairs of RAGS-related molecules and Eph RTKs participate in establishing the initial map of the retinotectal projection.

Experimental Procedures

Protein Purification and Analysis

Posterior thirds of chicken E9 and E10 tecta (including for technical reasons part of the ventrally situated structures [tegmentum]) were homogenized in 4 M ultrapure urea (Bethesda Research Laboratories), 10 mM spermidine in PBS in a dounce apparatus with loose-fitting piston. A volume of 50 μl per tectal third was used (all solutions contained 200 IU/ml aprotinin, 50 μM leupeptin, and 2 μM pepstatin). After incubation on ice for 1 hr, the suspension was pelleted at 16,000 × g for 30 min in a HB-4 rotor to remove unbroken cells and organelles. Membranes were collected by centrifuging the supernatant at 90 krpm for 15 min at 4°C in a Beckman TLA 100.3. The pellet was washed twice in PBS, then resuspended in 100 mM $NaCO_3$ (pH 11.5), and incubated on ice for 30 min. Membranes were pelleted at 90 krpm for 15 min, washed twice in 50 mM Tris (pH 7.4), and digested with PI-PLC (Boehringer, 0.1 U/mg protein) for 45 min at 37°C. After centrifugation at 90 krpm for 15 min, the supernatant containing PI-PLC-released proteins was concentrated using centricon-10 concentrators (Amicon). The remaining solution of ~50 μl was dried in a speed vac concentrator and washed repeatedly with 80% EtOH to remove any residual salts. The 2D analysis was done following the protocol of von Boxberg (1988). The separation in the electric charge dimension employed a nonequilibrium pH gradient electrophoresis.

Protein Sequencing

Internal sequences of the RAGS molecule were determined at Beckman Instruments. In brief, the protein spot at 25 kDa obtained from 2D gel electrophoresis was cut out and digested with LysC. After elution from the gel, individual peptides were HPLC separated on a Merck Supersher 60 RP select B column, and amino acid sequences were determined using a Beckman LF 3600 Sequencer.

Sequence Analysis

Nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using double-stranded DNA as template and T7 DNA polymerase. Both strands of the cDNA insert were sequenced after subcloning the cDNA insert as well as subfragments of it into pBluescript II (Stratagene). Sequence analysis was done using the Genetics Computer Group (GCG) software.

cDNA Library Construction and Screening

A randomly primed cDNA library was constructed from chicken E8 posterior tectum poly(A)$^+$ RNA by using the Librarian kit (Invitrogen). cDNAs were cloned into the eukaryotic expression vector pCDM8 (Invitrogen). Appoximately 400,000 clones from an amplified version of this cDNA library were screened using the following degenerate "guessmer" oligonucleotide: 5′-GAA/G CGI TAC/T GTI CTI TAC/T ATG GTI AAC/T TTC/T GAC/T GGI TA (I = inosine) derived from the peptide sequence ERYVLYMVNFDGY (see Figure 3A). The oligonucleotide was labeled with [γ-^{32}P]ATP using polynucleotide kinase (Sambrook et al., 1989). Hybridization to filters was done in 6 × SSC, 5 × Denhardt's, 0.1% SDS, 50 mM sodium phosphate (pH 6.8), 1 mM sodium pyrophosphate, 50 μg/ml tRNA at 45°C overnight. After hybridization, filters were washed finally in 6 × SSC, 0.1% SDS at 45°C and exposed to Kodak XAR-5 film with an intensifier for 4 days. Ten positive clones were obtained, replated, and rescreened twice to get individual colonies.

In Situ Hybridization

The full-length cDNA of RAGS was cloned into pBluescript II (Stratagene), which allows transcription from either side of the polylinker region. Labeling of RNA probes with digoxigenin-11-UTP was performed according to the recommendations of the manufacturer. Sections of 16–18 μm from fresh-frozen tecta were cut on a cryostat. The tissue was dried at 40°C and fixed for 10 min in 4% paraformaldehyde (PFA), washed in PBS, permeabilized for 10 min in 0.2 M HCl, washed in PBS, digested with proteinase K (1 μg/ml) at 37°C for 10 min, washed in PBS, and acetylated in 0.1 M triethanolamine (pH 8.0) for 10 min. Hybridization was done overnight at 60°C in 50% formamide, 5 × SSC, 5 × Denhardt's, 100 μg/ml yeast tRNA, 400 μg/ml torula RNA, and 3 ng/μl of digoxigenin-11-UTP-labeled probe. Transcripts of the sense strand were used as controls. After a final washing in 0.2 × SSC at 60°C, anti-digoxigenin Fab antibody was used to detect bound probe using established protocols (Schaeren-Wiemers and Gerfin-Moser, 1993).

Induction of a RAGS-Specific Antiserum

A 521 bp DNA fragment encoding the putative mature polypeptide of RAGS (amino acids 30–204) was generated by PCR and inserted in-frame into the bacterial expression vector pQE-12 (Crowe and Henco, 1992) providing the ATG start codon and a COOH-terminal His_6 tag. This modified RAGS protein was produced in Escherichia coli XL-1 blue and purified under denaturing conditions according to the instructions of the manufacturer. The purified protein was used for immunization of New Zealand White rabbits. The resulting antiserum was immunoabsorbed on an antigen column, and the purified polyclonal antibodies were used for immunoprecipitation.

^{35}S Labeling of COS Cells and Immunoprecipitation of RAGS

Human 293 cells were transfected with expression plasmids containing either the RAGS cDNA or a DNA fragment encoding β-galactosidase (see above). After transfection (24 hr), cells were labeled overnight in 5 ml of DMEM (without methionine and cysteine) plus 0.5% dialyzed FCS plus 500 μCi of Tran ^{35}S-label (ICN Biochemicals). After a 2 hr chase period in complete medium, cells were washed twice in PBS and finally incubated in 3 ml of PBS with or without addition of 1 U of PI-PLC for 1 hr at 37°C. Cells were harvested, and after ultracentrifugation, the resulting supernatant was concentrated to 50 μl and the pellet was resuspended in 50 μl. The samples were either directly diluted to 500 μl with TETN (25 mM Tris [pH 7.5], 5 mM EDTA, 250 mM NaCl, 1% Triton X-100, 0.5% Na-desoxycholate) or first denatured by adjusting to 1% SDS, boiled for 5 min, and then diluted to 500 μl with TETN. The following immunoprecipitation was done according to Firestone and Winguth (1990).

Collapse Assay and Stripe Assay

The procedure used for the collapse assay was essentially the same as that described by Cox et al. (1990). Retinal explants were grown overnight on a laminin surface in F12 culture medium plus 0.4% methylcellulose. At least 1 hr before application of the probe, the medium was replaced by F12 culture medium without methycellulose. Aliquots of sucrose membrane preparations of COS cells or tectal tissue (protein concentrations were determined according to Walter et al. [1987a]) were pelleted in an Eppendorf centrifuge and resuspended in 60 μl of F12 culture medium without methylcellulose. After sonication on ice (twice for 15 s at 30 W, Branson sonifier) probes were carefully applied to the retinal explants. Axonal growth cones were analyzed using a charge-coupled device (CCD) camera. By using a computer-controlled scanning stage (J. L., unpublished data), an average of 15 growth cones could be simultaneously observed in a single experiment by time-lapse. Pictures were taken under manual control every 1–3 min. They were digitized and stored on a computer hard disk. For analysis, the complete sequence was reloaded using the NIH Image 1.55 program.

The stripe assay experiments followed the protocol of Walter et al. (1987a) 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 hr at 37°C. Afterward, 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, dilutions were done using mock-transfected COS membranes.

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