

# Receptor Protein Tyrosine Phosphatases Regulate Retinal Ganglion Cell Axon Outgrowth in the Developing *Xenopus* Visual System

Karl G. Johnson,<sup>1\*</sup> Iain W. McKinnell,<sup>2</sup> Andrew W. Stoker,<sup>2</sup> Christine E. Holt<sup>1</sup>

<sup>1</sup> Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, United Kingdom

<sup>2</sup> Neural Development Unit, Institute of Child Health, University College London, London, WC1N 1EH, United Kingdom

Received 21 March 2001; accepted 30 May 2001

**ABSTRACT:** Receptor protein tyrosine phosphatases (RPTPs) are regulators of axon outgrowth and guidance in a variety of different vertebrate and invertebrate systems. Three RPTPs, CRYP- $\alpha$ , PTP- $\delta$ , and LAR, are expressed in overlapping but distinct patterns in the developing *Xenopus* retina, including expression in retinal ganglion cells (RGCs) as they send axons to the tectum (Johnson KG, Holt CE. 2000. Expression of CRYP-alpha, LAR, PTP-delta, and PTP-rho in the developing *Xenopus* visual system. *Mech Dev* 92:291–294). In order to examine the role of these RPTPs in visual system development, putative dominant negative RPTP mutants (CS-CRYP- $\alpha$ , CS-PTP- $\delta$ , and CS-LAR) were expressed either singly or in combination in retinal cells.

No effect was found on either retinal cell fate determination or on gross RGC axon guidance to the tectum. However, expression of these CS-RPTP constructs differentially affected the rate of RGC axon outgrowth. *In vivo*, expression of all three CS-RPTPs or CS-PTP- $\delta$  alone inhibited RGC axon outgrowth, while CS-LAR and CS-CRYP- $\alpha$  had no significant effect. *In vitro*, expression of CS-CRYP- $\alpha$  enhanced neurite outgrowth, while CS-PTP- $\delta$  inhibited neurite outgrowth in a substrate-dependent manner. This study provides the first *in vivo* evidence that RPTPs regulate retinal axon outgrowth. © 2001 John Wiley & Sons, Inc. *J Neurobiol* 49: 99–117, 2001

**Keywords:** RPTP; outgrowth; guidance; retina; tectum

## INTRODUCTION

The retinotectal system has served as a model for the growth and navigation of axons in the developing brain for over 50 years, when the first anatomical studies revealed details of the ontogeny of this pathway (Herrick, 1941). Within the past 20 years, numerous molecules have been identified at several

choice points that help guide axons along the appropriate route, but in no case has a particular guidance choice been completely characterized at the molecular level. Nevertheless, the development of the retinotectal projection is possibly the best understood case of axonal navigation, both anatomically and molecularly, from origin to final target.

The regulation of tyrosine phosphorylation plays a critical role in the development of the visual system, demonstrated by the presence of multiple protein tyrosine kinases (PTKs) involved in guiding retinal ganglion cell (RGC) axons along the optic pathway to their topographically appropriate targets in the tectum. Normal function of the fibroblast growth factor (FGF) family of receptor protein tyrosine kinases

\* Present address: Department of Cell Biology, Harvard Medical School, Building LHRB 409, 270 Longwood Ave., Boston, MA 02115.

Correspondence to: K. G. Johnson (karl\_johnson@hms.harvard.edu).

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(RPTKs) is required for proper tectal target recognition (McFarlane et al., 1995, 1996) and retinal cell fate determination in *Xenopus* (McFarlane et al., 1998). The EphB family of RPTKs regulate crossing at the *Xenopus* optic chiasm (Nakagawa et al., 2000), while the graded expression of the EphA family of RPTKs establishes anterior-posterior topography of retinal axons in the chick optic tectum (Cheng et al., 1995; Frisen et al., 1998; Nakamoto et al., 1996). In addition, cytoplasmic tyrosine kinases promote RGC axonogenesis (Worley et al., 1997) and axon extension along the *Xenopus* optic tract (Worley and Holt, 1996).

Recent studies have shown that receptor protein tyrosine phosphatases (RPTPs), most notably those of the type IIa subfamily, are involved in axon guidance and outgrowth. Type IIa RPTPs have cell adhesion molecule-like extracellular domains and two cytoplasmic phosphatase domains (reviewed in Bixby, 2000; Stoker, 2001). In *Drosophila*, type IIa RPTPs regulate motor axon guidance and target recognition (Desai et al., 1996, 1997; Krueger et al., 1996; Sun et al., 2000), photoreceptor axon outgrowth (Garrity et al., 1999; Newsome et al., 2000), and midline crossing (Sun et al., 2000), while in leech, the proper function of an LAR homologue is required for the patterned outgrowth of comb cell processes (Baker and Macagno, 2000; Baker et al., 2000; Gershon et al., 1998a,b). Recently, another type IIa RPTP, CRYP- $\alpha$  (the orthologue of mammalian PTP- $\sigma$ ), has been implicated in promoting intraretinal RGC axon outgrowth in chick (Ledig et al., 1999a). However, these studies have not yet led to a detailed model of how this family of RPTPs is involved in RGC axon outgrowth and guidance.

All three known members of the type IIa RPTP subfamily, CRYP- $\alpha$ , PTP- $\delta$ , and LAR, are expressed in overlapping but distinct patterns in the developing *Xenopus* retina, including expression in retinal ganglion cells during periods of axon navigation from the retina to the tectum (Johnson and Holt, 2000), suggesting that these RPTPs may be involved in RGC axon guidance, axon outgrowth, or cell fate determination. Therefore, we sought to determine the role that type IIa RPTPs play in RGCs by expressing putative dominant negative forms of CRYP- $\alpha$ , PTP- $\delta$ , and LAR and analyzing the effects on retinal development both *in vitro* and *in vivo*. Our results suggest that two of these type IIa RPTPs, CRYP- $\alpha$  and PTP- $\delta$ , regulate the rate of RGC axon outgrowth from the retina to the tectum, but do not appear to influence retinal cell fate decisions or RGC axon guidance.

## MATERIALS AND METHODS

### *Xenopus* Embryos

Eggs were obtained from human chorionic gonadotropin (Sigma) stimulated *Xenopus laevis* females and were fertilized *in vitro* with sperm harvested from *Xenopus laevis* males. Embryos were reared in 0.1 $\times$  MBS pH 7.4 [0.88 mM NaCl, 10  $\mu$ M KCl, 24  $\mu$ M NaHCO<sub>3</sub>, 100  $\mu$ M HEPES, 8.2  $\mu$ M MgSO<sub>4</sub>, 3.3  $\mu$ M Ca(NO<sub>3</sub>)<sub>2</sub>, 4.1  $\mu$ M CaCl<sub>2</sub>]. The jelly coat was removed by treatment with 2% cysteine in 0.1 $\times$  MBS at pH 7.5–8.0. Embryos were repeatedly washed with 0.1 $\times$  MBS after cysteine treatment, and were reared at temperatures ranging from 14–24°C.

### CS-RPTP Construction

cDNAs encoding the entire cytoplasmic domains of CRYP- $\alpha$ , PTP- $\delta$ , and LAR (lacking 50 membrane-proximal amino acids) were used as templates for site-directed mutagenesis. A highly conserved cysteine in the first phosphatase domain that is essential for catalytic activity was changed into a serine using the QuikChange Site-Directed Mutagenesis Kit. Such C-S mutants in the first phosphatase domain of LAR abolish more than 99% of phosphatase activity (Pot et al., 1991) while retaining the ability to bind substrate (Furukawa et al., 1994; Milarski et al., 1993). These C-S mutant constructs were myc tagged (MT) at the 5' end and were subcloned into pCS2+ for expression studies.

### Expressing CS-RPTP Constructs

pCS2+MT+CS-RPTP plasmids were diluted to 50 ng/ $\mu$ L in sterile water and were injected into the blastomeres of 4–16 cell stage *Xenopus* embryos that would give rise to the retinas. DNA blastomere injections were conducted using 10 nL of a 1:1 ratio of GFP-myc:CS-RPTP construct, resulting in a total of 250 pg of CS-RPTP construct and 250 pg of GFP-myc per injected blastomere. Injected embryos were reared in 0.1 $\times$  MBS.

To introduce constructs into the retinal neuroepithelium *in vivo*, DNA lipofections were conducted. CS-RPTP DNA was mixed in a 1:1 ratio with GFP-myc, and used at a 1  $\mu$ g:3  $\mu$ L dilution in DOTAP as previously described (Dorsky et al., 1995; Holt et al., 1990; Lilienbaum et al., 1995; McFarlane et al., 1996). Ten nanoliters of the DNA-DOTAP mixtures was injected into the developing optic vesicle at stage 19.

### Immunohistochemistry

Cryostat sections of paraformaldehyde fixed *Xenopus* embryos were cut at 15–25  $\mu$ M thickness on a cryostat and dried for 30 min at room temperature. A hydrophobic border was drawn around the dried sections using a PAP pen (The Binding Site, Inc.) and the slides were rinsed 2  $\times$  5 min in

PBS and immersed in 100% MeOH at  $-20^{\circ}\text{C}$ . Slides were washed  $5 \times 5$  min in  $1 \times$  PBS and blocked for 2 h in 4% skim milk in PBS. The antimyc primary antibody 9E10 (Sigma) was added at a 1:1000 dilution in 4% skim milk in PBS for 2 h at room temperature. Primary antibody was washed off  $5 \times 5$  min in PBS and the slides were incubated in a 1:500 dilution of Cy-3 conjugated donkey antimouse IgG secondary antibody (Jackson ImmunoResearch) for 1 h at room temperature in a dark moist chamber. Secondary antibody was washed off  $5 \times 5$  min in PBS, and sections were mounted in Fluosave + DABCO.

For immunohistochemistry on wholemount brains, fixed stage 35/36–41 embryos were pinned to a sylgard lined 35 mm Petri dish and brains were dissected free. Isolated brains were placed in PBS + 0.5% Triton in an eppendorf tube. Brains were washed  $5 \times 15$  min in PBS + 0.5% Triton and blocked for 4–12 h in 5% HIGS in PBS + 0.5% Triton. Primary antibody 9E10 was added at a 1:2000 dilution for an overnight rocking incubation at  $4^{\circ}\text{C}$ . Primary antibody was washed off with  $6 \times 60$  min washes in PBS + 0.5% Triton, and secondary antibody (Cy-3 conjugated donkey antimouse) was added at a 1:1000 dilution in 5% HIGS + PBS + 0.5% Triton for an overnight rocking incubation at  $4^{\circ}\text{C}$ . Secondary antibody was washed off with  $6 \times 60$  min washes in PBS + 0.5% Triton and a final rinse in  $1 \times$  PBS. Brains were mounted on Superfrost Plus microscope slides in Fluosave + DABCO inside two clear stacked reinforcement rings.

## Retinal Transplants

Embryos received DNA blastomere injections with 250 ng (each) of CS-CRYP- $\alpha$  + GFP-myc, CS-PTP- $\delta$  + GFP-myc, CS-LAR + GFP-myc, CS-H3P (250 ng of each CS-RPTP) + GFP-myc, or GFP-myc alone. Stage 24 DNA-blastomere injected embryos were examined for GFP expression in the developing optic vesicles. Embryos with high levels of expression were transferred to a clay-bottomed 35 mm Petri dish with  $1 \times$  MBS + 0.05% MS222 + 0.05% BSA. The optic vesicle and the overlying skin was carefully dissected off and transplanted into a host stage 24 embryo (from which the corresponding optic vesicle had been removed) by carefully aligning the severed optic stalks. A small piece of coverslip glass was placed over the transplanted retina and these embryos were allowed to heal for 30 min in  $1 \times$  MBS + .05% MS222 + .05% BSA. Embryos were then transferred to  $1 \times$  MBS for 1 h, then were grown in  $0.1 \times$  MBS to stage 39–41. Immunohistochemistry was performed as on wholemount brains as described above.

## Retinal Explant Cultures

Poly-L-lysine (PLL) coated coverslips were coated with chick retinal basement membranes  $\pm$  glial endfeet as previously described (Halfter et al., 1987). Coated coverslips were placed in 4-well dishes with 0.5 mL of culture media {60% L15, 40%  $\text{H}_2\text{O}$ , 0.1% BSA, 0.4% methyl cellulose, plus antibiotics/antimycotics [100 U/mL penicillin G so-

dium, 100 ug/mL streptomycin sulfate, and 0.25 ug/mL amphotericin B in 0.85% saline (GibcoBRL)] pH 7.6}. Stage 19 DNA-blastomere injected embryos were screened for GFP expression in the developing optic vesicles using a fluorescent dissecting microscope (Leica MZ FLIII). Embryos expressing GFP were collected and grown to stage 28 and the fluorescent retinas were dissected free and divided into four to six pieces. Retina explants were plated on coated coverslips and were grown for 20 h at room temperature. Immunohistochemistry was performed as for cryostat sections, as described above.

## RESULTS

### Construction and Expression of Putative Dominant Negative RPTPs

Site-directed mutagenesis was used to convert the catalytically essential cysteine into a serine in the first phosphatase domain of CRYP- $\alpha$ , PTP- $\delta$ , and LAR. C-S mutants in the first phosphatase domain have been shown to abolish more than 99% of enzymatic activity (Pot et al., 1991), while still retaining the ability to bind substrate (Furukawa et al., 1994; Milarski et al., 1993). The cytoplasmic domains of these C-S mutant constructs were cloned in frame into pCS2 + 6 Myc tags, and were transcribed and translated *in vitro* (Fig. 1). Electrophoretic analysis of translation products revealed single bands of the appropriate size (approximately 80 kDa) for each of these constructs (data not shown). Western blots of total protein isolated from DNA-blastomere injected embryos showed that *Xenopus* embryos can synthesize myc-tagged CS-RPTP fusion proteins from constructs injected at the two-cell stage by stage 19 (Fig. 1). Myc-RPTP fusion proteins (as well as GFP-myc) are expressed until at least stage 41 (data not shown).

To facilitate the analysis of the effects of CS-RPTP expression on cell fate determination, the coexpression efficiency of CS-RPTP constructs with GFP at stages 33/34–41 was determined following DNA blastomere injections at the eight-cell stage. Efficiency of coexpression at stage 41 was also examined following *in vivo* lipofection of DNA into the retina at stage 19. High coexpression efficiency occurs following both DNA blastomere injections (98–99% coexpression) and DNA lipofections (80–90% coexpression). Therefore, if a particular cell from a DNA-blastomere injected embryo is positive for GFP, there is a 98–99% chance that it also expresses the CS-RPTP construct, while 80–90% of cells expressing GFP following DNA lipofections also express the CS-RPTP construct (data not shown). In addition, in an embryo receiving a DNA blastomere injection with

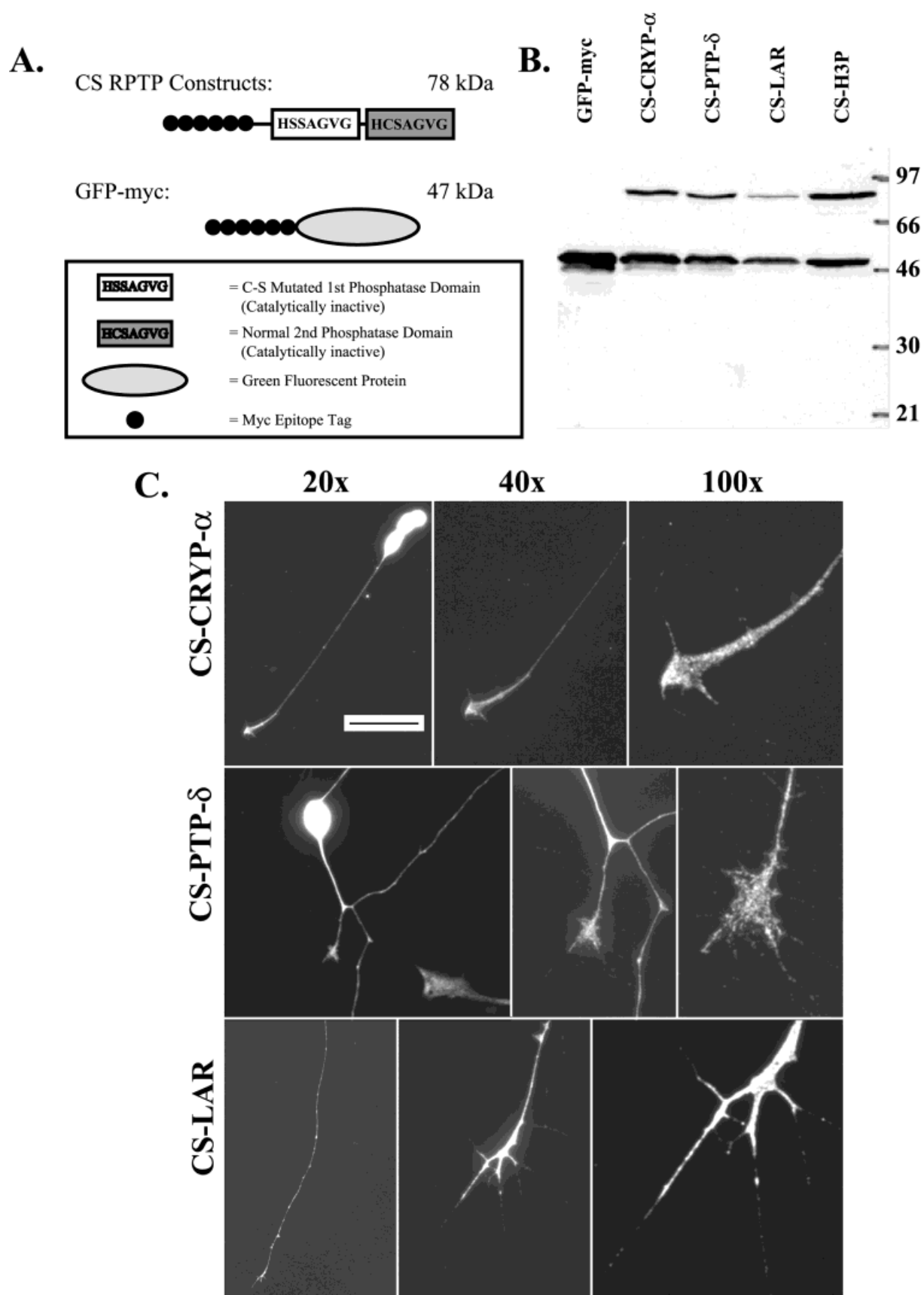


Figure 1

CS-CRYP- $\alpha$ , CS-PTP- $\delta$ , CS-LAR, and GFP, a GFP-positive cell has a 96% probability of also expressing all three CS-RPTP constructs. Therefore, counting GFP-expressing cells in GFP/CS-RPTP coinjected embryos should accurately reflect the effects of CS-RPTP expression on cell fate determination.

In order to determine if the CS-RPTP constructs were transported to the same subcellular location as the endogenous protein, CS-RPTP protein expression was analyzed *in vitro*. Embryos injected with 250 pg of CS-CRYP- $\alpha$ +GFP, CS-PTP- $\delta$ +GFP, and CS-LAR+GFP were reared to stage 28. Retinas expressing GFP were dissected free from the embryo, dissociated, and cultured for 24 h on laminin (10  $\mu$ g/mL). Anti-myc antibody staining revealed robust expression of each CS-RPTP in the cell body, neurite, and growth cone when grown on laminin [Fig. 1(C)]. Because endogenous type IIa RPTPs have been previously shown to be concentrated in growth cones (Stoker et al., 1995; Zhang et al., 1998), the CS-RPTP constructs appear to be expressed in the appropriate subcellular location to block the function of the endogenous RPTP.

### CS-RPTP Expression Has No Effect on Retinal Cell Fate

CRYP- $\alpha$ , PTP- $\delta$ , and LAR are expressed in spatially and temporally distinct patterns in the developing retina during periods of retinal differentiation (Johnson and Holt, 2000). CRYP- $\alpha$  is expressed throughout the ganglion cell layer (GCL) and the inner nuclear layer (INL), LAR is expressed in the GCL and lens proximal half of the INL [presumably in amacrine cells (Ramon y Cajal, 1972)], and PTP- $\delta$  is expressed in the GCL and lens distal half of the INL [presumably in bipolar cells (Ramon y Cajal, 1972)]. Therefore, the possibility that these RPTPs are involved in cell fate determination was explored.

DNA blastomere injections were conducted using 250 pg (each) of CS-CRYP- $\alpha$ +GFP, CS-PTP- $\delta$ +GFP, and CS-LAR+GFP, CS-L3P (a low concentration, 83 pg each, of all three CS-RPTPs), CS-H3P

(a high concentration, 250 pg each, of all three CS-RPTPs), and GFP alone. GFP-positive cells were counted in sections of stage 40–41 retinas, and assigned a particular cell fate based on the position of their cell body and the morphology of processes as described previously (Dorsky et al., 1995, 1997; McFarlane et al., 1998; Ohnuma et al., 1999). None of the CS-RPTP constructs caused significant changes in the percentage of any particular cell type (Fig. 2), suggesting that these RPTPs are not involved in cell fate determination in the *Xenopus* retina.

### CS-RPTP Expression Does Not Affect RGC Axon Guidance *in Vivo*

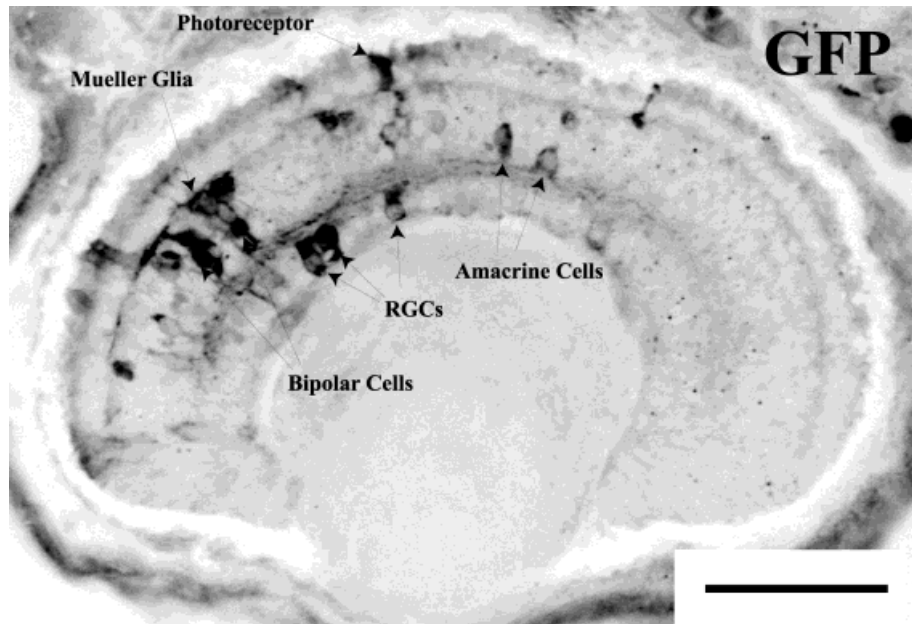
Because CS-RPTP constructs are concentrated in the growth cone of RGC axons *in vivo* but are not strongly present along axons (data not shown), and because there is a high level of CS-RPTP/GFP coexpression, GFP-myc coexpression was used to increase the antimyc signal intensity and to label CS-RPTP-expressing RGCs from the cell body to the growth cone. *In vitro*, CRYP- $\alpha$  has been shown to regulate the rate of axon outgrowth and the morphology of RGC growth cones (Ledig et al., 1999a), but the roles of type IIa RPTPs in RGC axon guidance *in vivo* have not been examined. In order to determine if RGC axons expressing CS-RPTP constructs could navigate from the retina to the tectum, embryos were lipofected with CS-RPTP constructs, grown to stage 40, sectioned, and stained. Sectioned stage 40 retinas and brains showed that RGCs expressing CS-RPTPs+GFP-myc can navigate from the retina to the developing tectum (Fig. 3). All embryos with CS-RPTP-expressing RGCs in the retina also had CS-RPTP-expressing RGC axons in the contralateral tectum (GFP-myc,  $n = 17$ ; CS-CRYP- $\alpha$ ,  $n = 17$ ; CS-PTP- $\delta$ ,  $n = 16$ ; CS-LAR,  $n = 16$ ) but not in the ipsilateral tectum, or in any positions indicative of guidance defects.

Additionally, embryos that received transplanted retinas expressing CS-CRYP- $\alpha$ +GFP-myc, CS-PTP- $\delta$ +GFP-myc, CS-LAR+GFP-myc, CS-H3P (all three

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**Figure 1** CS-RPTP constructs are expressed in retinal growth cones *in vitro*. (A) Schematic diagram of the myc-tagged CS-RPTP constructs and the myc-tagged GFP construct used in this study. (B) Antimyc Western blot of total protein isolated from stage 19 embryos that received a DNA blastomere injection with CS-RPTP construct(s) and/or GFP-myc. Myc-CS-RPTP fusion proteins (80 kDa) and GFP-myc (50 kDa) are both expressed at this stage. (C) Anti-myc stained cultures of retinas expressing CS-RPTPs+GFP reveal CS-RPTP protein expression in the cell body, axon, and growth cone (including filopodial processes) of retinal neurites. Scale bar = 50  $\mu$ m at 20X magnification.





Construct:	Photoreceptor	Horizontal	Mueller Glia	Bipolar	Amacrine	Ganglion	N
GFP	23.28%	2.08%	1.82%	41.61%	10.27%	20.94%	769
CS-CRYP- $\alpha$	22.66%	1.42%	1.98%	43.34%	11.05%	19.55%	353
CS-PTP- $\delta$	21.23%	0.56%	1.40%	49.16%	9.78%	17.88%	358
CS-LAR	21.38%	1.32%	2.63%	43.42%	10.53%	20.72%	304
CS-L3P	23.66%	1.15%	1.53%	41.22%	11.83%	20.61%	262
CS-H3P	20.59%	1.47%	1.47%	43.38%	11.76%	21.32%	272

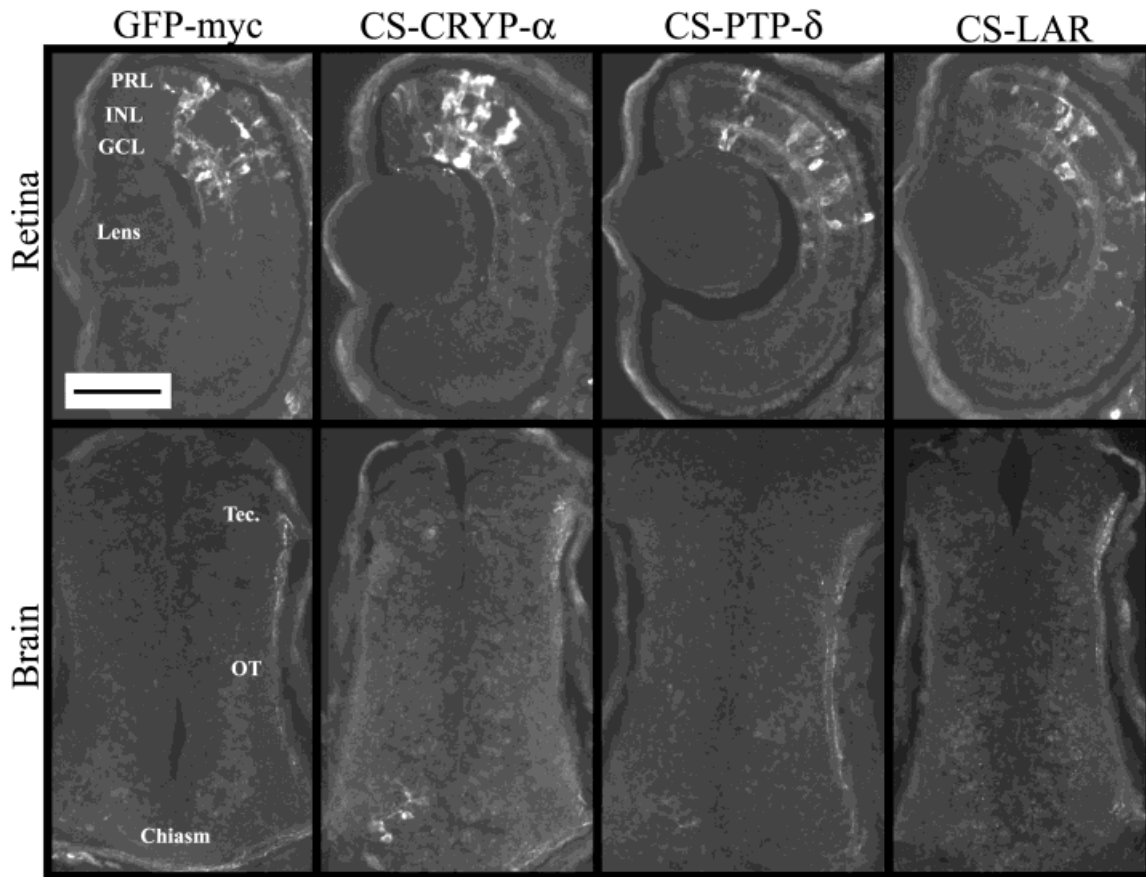
**Figure 2** CS-RPTP expression has no effect on retinal cell fate determination. GFP-expressing retinal cells were assigned to a particular cell fate based on the position of the cell body and the direction of process outgrowth. A section through the retina of a GFP-injected embryo shows many different cell types. The percentage of GFP-positive cells of each cell type was not affected by coexpression of CS-RPTP constructs. CS-L3P, coexpression of a low concentration of all three CS-RPTP constructs; CS-H3P, coexpression of a high concentration of all three CS-RPTP constructs. Scale bar = 50  $\mu$ m.

CS RPTPs)+GFP-myc, or GFP-myc alone, also demonstrated that there are no major pathfinding errors exhibited by RGC axons expressing CS-RPTP constructs in the optic tract (Fig. 4). Target recognition defects or tectal bypass phenotypes previously characterized in this system following perturbations of FGFR signaling (McFarlane et al., 1996) were not seen. Rather, RGC axon guidance toward the tectum appeared unaffected by the expression of one or multiple type IIa CS-RPTP constructs.

#### **RGCs Expressing CS-PTP- $\delta$ Alone, or All Three CS-RPTPs Together, Have Shorter Axons *in Vivo***

Tyrosine phosphorylation is a key regulator of RGC axon outgrowth. Perturbations of either cytoplasmic

(Worley and Holt, 1996) or receptor (McFarlane et al., 1996) PTK function can significantly decrease the rate of RGC axon outgrowth in the developing *Xenopus* visual system. In order to determine if overexpression of multiple CS-RPTP constructs could alter the rate of RGC axon outgrowth, retinas expressing all three CS-RPTPs (CS-H3P)+GFP-myc or GFP-myc alone were transplanted into host embryos, and RGC axon outgrowth was analyzed in wholemount brains. Transplantation of retinas overexpressing CS-H3P+GFP-myc and GFP-myc alone into uninjected embryos revealed that CS-H3P expression increases the percentage of RGC growth cones in the optic tract at stage 40 (Fig. 5). Approximately 40% of RGC axons expressing CS-H3P had growth cones in the optic tract, compared with only 23% of RGC axons expressing GFP-myc (Table 1).

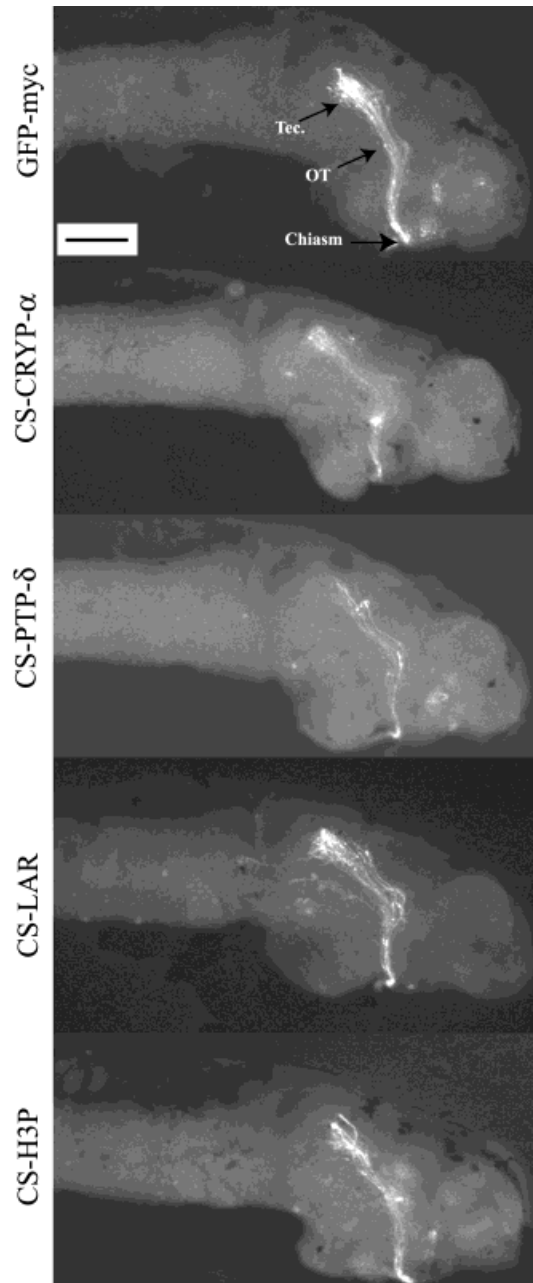


**Figure 3** Axon guidance in the retina and optic pathway is not perturbed by CS-RPTP expression. RGC axon outgrowth and guidance were analyzed on 15  $\mu\text{m}$  coronal cryostat sections through stage 40 CS-RPTP lipofected embryos. Retinae were lipofected with CS-CRYP- $\alpha$ +GFP-myc, CS-PTP- $\delta$ +GFP-myc, CS-LAR+GFP-myc, or GFP-myc alone at stage 19. Antimyc staining revealed that all embryos with RGCs expressing these constructs had myc-positive axons in the tectum, suggesting that there were no catastrophic defects in RGC axonogenesis, outgrowth, or guidance following the overexpression of CS-RPTPs. GCL, ganglion cell layer; INL, inner nuclear layer; PRL, photoreceptor layer; OT, optic tract; Tec, tectum. Scale bar = 50  $\mu\text{m}$ .

In order to perform a statistical analysis on the effect of CS-RPTP expression on RGC axon outgrowth *in vivo*, the positions of RGC growth cones were grouped into one of six equal bins (approximately  $50 \times 50 \mu\text{m}$ ) according to their position along the optic tract [see Fig. 6(A)]. Chi-squared tests comparing the distribution of growth cones in these bins showed that at stage 40, axons expressing CS-H3P+GFP-myc had a significantly different distribution in the six bins than axons expressing GFP-myc alone ( $p < .0001$ ) (Table 1). To determine if axons expressing CS-H3P were significantly shorter, a  $\chi^2$  test was conducted on a  $2 \times 2$  table comparing the number of growth cones expressing GFP-myc with the number of growth cones expressing CS-H3P, in the first (bins 1–3) and second (bins 4–6) halves of the optic pathway. This analysis demonstrated that

significantly more CS-H3P-expressing RGC axons were present in the ventral part of the optic tract, and therefore that CS-H3P-expressing RGC axons were significantly shorter than GFP-myc-expressing axons *in vivo* ( $p < .0001$ ).

In order to determine if the expression of an individual CS-RPTP could cause a reduction in RGC axon length, outgrowth was analyzed in wholemount brains following lipofection of individual CS-RPTP constructs at stage 19. Normally, the first RGC axons reach the optic chiasm at stage 32, can be visualized in the optic tract at stage 33/34, and reach the tectum at stage 37/38 (Holt, 1984). Because the majority of axons have reached the tectum by stage 40 (Holt, 1984), the positions of RGC growth cones within the optic tract at stages 35/36, 37/38, 39, and 40 were analyzed. CS-RPTP-expressing growth cones were



**Figure 4** RGC axons expressing CS-RPTP constructs, either individually or in combination, navigate correctly to the tectum. Retinas from stage 26 embryos that had received DNA blastomere injections with CS-CRYP- $\alpha$ +GFP-myc, CS-PTP- $\delta$ +GFP-myc, CS-LAR+GFP-myc, CS-H3P+GFP-myc, or GFP-myc alone were transplanted into stage 26 hosts and grown to stage 41. No obvious pathfinding defects were made by axons expressing these CS-RPTP constructs. OT, optic tract; Tec, tectum; CS-H3P, coexpression of all three CS-RPTP constructs. Scale bar = 100  $\mu$ m.

grouped into one of six bins according to their dorsal-ventral position in the optic tract [Fig. 6(A)]. Chi-squared tests comparing RGC axons expressing CS-

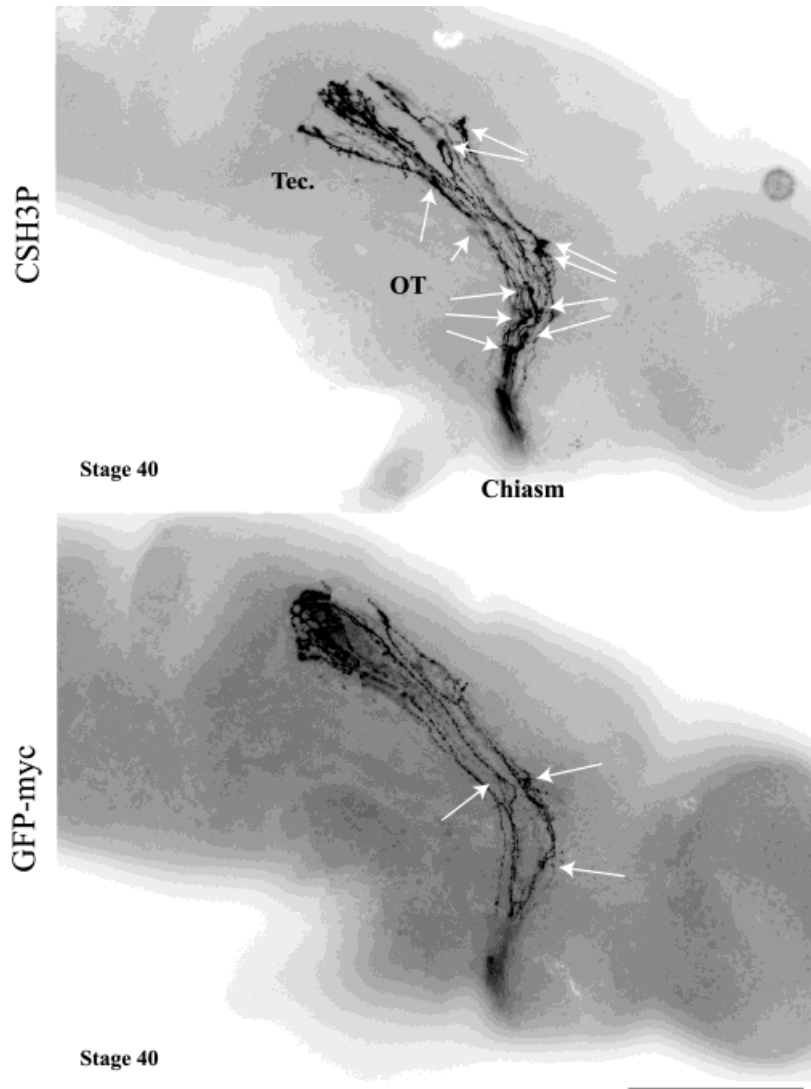
RPTPs+GFP-myc with axons expressing GFP-myc alone showed that at stage 39, cells expressing CS-PTP- $\delta$  had a significantly different distribution of axons than cells expressing GFP-myc alone (Fig. 6 and Table 2). To determine if axons expressing CS-PTP- $\delta$  were significantly shorter, a  $\chi^2$  test was conducted comparing the numbers of growth cones expressing GFP-myc with the numbers of growth cones expressing CS-PTP- $\delta$  in the first (bins 1–3) and second (bins 4–6) halves of the optic pathway. This analysis demonstrated that significantly more CS-PTP- $\delta$ -expressing RGC axons were present in the ventral part of the optic tract, and therefore that CS-PTP- $\delta$ -expressing RGC axons were significantly shorter than GFP-myc-expressing axons *in vivo* (Table 3).

### CRYP- $\alpha$ and PTP- $\delta$ Influence Neurite Outgrowth *in Vitro*

CRYP- $\alpha$  is expressed by RGCs (Johnson and Holt, 2000; Ledig et al., 1999b), while its ligand is expressed on retinal basement membranes in chick (Haj et al., 1999; Ledig et al., 1999a,b) and in *Xenopus* (data not shown). PTP- $\delta$ , which is a homophilic neurite outgrowth promoting cell adhesion molecule (Wang and Bixby, 1999) is expressed by RGCs (Johnson and Holt, 2000) and likely by retinal Mueller glia (Johnson and Holt, 2000; Shock et al., 1995). Blocking CRYP- $\alpha$  ligand-receptor interactions, either at the ligand or receptor level, can inhibit axon outgrowth on retinal basement membranes (Ledig et al., 1999a). Thus, receptor-ligand interactions for both CRYP- $\alpha$  and PTP- $\delta$  have previously been shown to increase the rate of axon outgrowth. In order to determine if putative dominant negative RPTPs can mimic the effects of blocking receptor-ligand interactions already characterized *in vitro* (Ledig et al., 1999a), the effects of overexpressing these CS-RPTPs on *in vitro* outgrowth were determined.

Retinal explants expressing CS-RPTP constructs were cultured on chick retinal basement membranes with and without glial endfeet. Because Mueller glia express CRYP- $\alpha$  and probably PTP- $\delta$  ligands (Ledig et al., 1999a; Shock et al., 1995), these experiments were conducted in the presence and absence of glial endfeet, to determine if the effects of CS-RPTP expression were dependent on the presence of proteins associated with glial endfeet (such as the CRYP- $\alpha$  ligand, and possibly the PTP- $\delta$  ligand). Expression of CS-CRYP- $\alpha$ +GFP-myc significantly increased neurite length on basement membranes with glial endfeet, while expression of CS-PTP- $\delta$ +GFP-myc reduced the mean neurite length on basement membranes with glial endfeet. There were no





**Figure 5** Expression of all three CS-RPTPs increases the number of RGC axons with growth cones in the optic tract. Eleven growth cones are present in the optic tract of brains that received CS-H3P transplants, while only three growth cones are present in the optic tract of brains that received GFP-myc transplants. Images shown are assembled composites of four to eight different focal planes following anti-myc staining on wholemount brains. Images are inverted black and white images of fluorescently labeled axons. Dorsal is up, anterior is to the right. OT, optic tract; Tec, tectum; CS-H3P, coexpression of all three CS-RPTP constructs. Scale bar = 100  $\mu$ m.

significant changes in axon length for any of these constructs in the absence of glial endfeet, suggesting that these CS-RPTP constructs may regulate axon outgrowth only in the presence of their endogenous RPTP ligands (Table 4 and Fig. 7).

## DISCUSSION

We have demonstrated that expression of CS-RPTPs influences RGC axon outgrowth in the developing

*Xenopus* visual system. Expression of CS-PTP- $\delta$  reduces the rate of RGC axon outgrowth by nearly 20% *in vitro* and *in vivo*, while expression of CS-CRYP- $\alpha$  increases neurite length by 50% *in vitro*. The *in vitro* effects are substrate dependent; following the removal of glial endfeet [a major site of CRYP- $\alpha$  ligand expression (Haj et al., 1999), and a likely site of PTP- $\delta$  ligand expression (Johnson and Holt, 2000; Shock et al., 1995)] the effects of these CS-RPTP constructs are abolished. These data suggest that the type IIa RPTPs are involved in regulating axon outgrowth, but

**Table 1** Distribution of CS-H3P and GFP-myc Expressing Axons in Bins along the Optic Tract

Bin Number	% of CS-H3P Expressing Growth Cones in Bin	% of GFP-myc Expressing Growth Cones in Bin
1 (Near chiasm)	6.5	2.8
2	9.0	3.4
3	7.6	5.2
4	7.1	4.4
5	8.5	7.6
6 (Optic tectum)	61.3	76.7
<i>n</i> =	354	502

Chi-squared test on  $2 \times 2$  table:  $p$ -value =  $3.96 \times 10^{-6}$ .  
RGCs expressing all three CS-RPTP constructs more frequently have growth cones in the optic tract than RGCs expressing GFP-myc alone.

not in controlling RGC axon guidance or cell fate decisions in the developing *Xenopus* visual system.

**Mechanism of CS-RPTP Action**

The CS-RPTPs used in this study were likely functioning as dominant negative proteins. There are two lines of evidence that suggest this is the case. First, expression of a full-length CS-PTP- $\mu$  has been shown to function as a dominant negative construct in the developing chick visual system (Burden-Gulley and Brady-Kalnay, 1999). In this study, expression of a full-length PTP- $\mu$  construct with a C-S mutation in the first phosphatase domain inhibited neurite outgrowth *in vitro* to a similar degree and in a similar substrate-dependent manner as antisense PTP- $\mu$  RNA (Burden-Gulley and Brady-Kalnay, 1999). This suggests that the catalytic activity of the first phosphatase domain is essential for signaling, and that expressing a construct that lacks this catalytic activity results in a loss-of-function phenotype. Second, overexpression of the cytoplasmic phosphatase domains of Dlar can mimic the Dlar mutant phenotype in *Drosophila* (Wan and Goodman, 1997), demonstrating that the expression of a cytoplasmic construct can also inhibit the function of the endogenous RPTP, presumably by competing for endogenous substrates. While it is likely that overexpressing the cytoplasmic domains of RPTPs would function as a dominant negative in the developing *Xenopus* visual system, we elected to use cytoplasmic C-S mutant constructs to ensure that our constructs would not have catalytic activity.

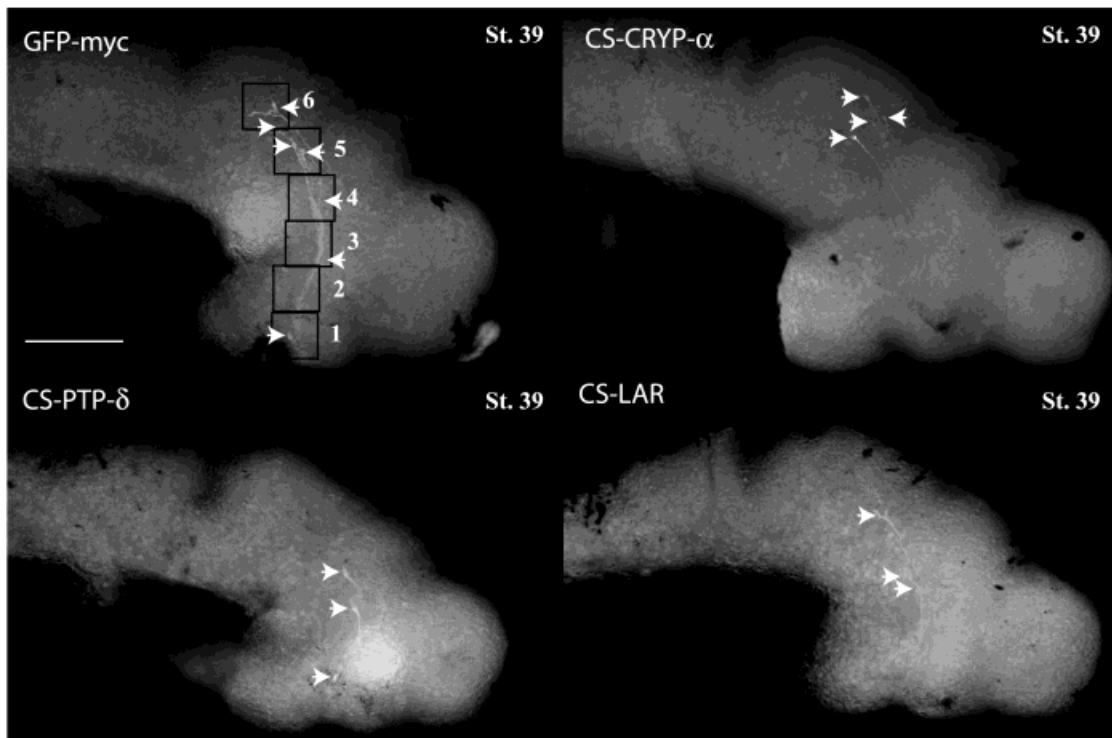
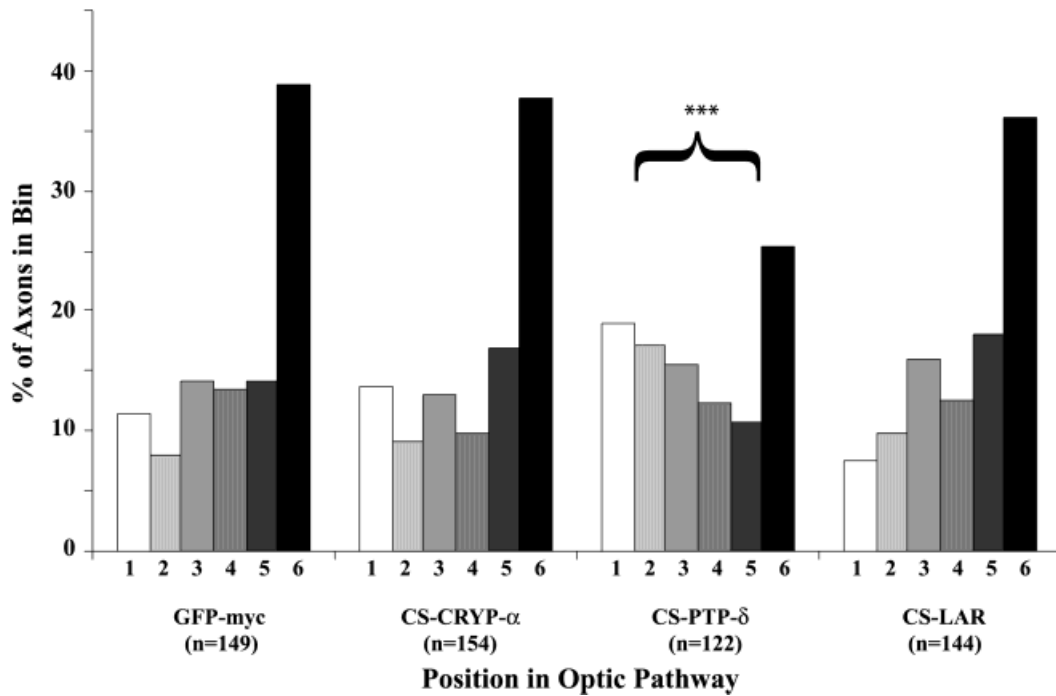
It is possible, however, that these constructs were functioning as specific dominant positives. C-S mutant proteins may bind to and titrate out the effect of an endogenous inhibitor of RPTPs, or, the expression of such constructs may facilitate a catalysis-independent mechanism of RPTP action (such as docking). However, while there are other possible explanations for the results

seen in this study, the fact that similar constructs have been shown to function as dominant negatives in other systems provides support for a dominant negative function of these constructs in this system. Furthermore, because the effects of CS-CRYP- $\alpha$  and CS-PTP- $\delta$  expression appear to be dependent on the presence of RPTP ligand(s), a dominant positive effect seems unlikely, as such an effect would occur both in the presence and in the absence of ligand(s).

It is essential to state, however, that we cannot be sure of the mechanism by which these CS-RPTP constructs are acting. In the studies described above, one used a full-length C-S construct to function as a dominant negative, while the other study is made up of unpublished observations based on Dlar in *Drosophila*. The constructs used in this study, therefore, may not necessarily be functioning as dominant negatives. Unfortunately, because the ligands and substrates for these RPTPs are not known, there is currently no conclusive way to demonstrate whether they function as dominant negative constructs to specifically inhibit the function of the endogenous protein. These CS-RPTP constructs, for example, may bind and inhibit another RPTP [mimicking the heterodimerization previously demonstrated for PTP- $\delta$  and CRYP- $\alpha$  (Wallace et al., 1998)], or they may have more complex relationships than direct inhibition of the endogenous protein. Thus, although previous studies suggest these may function as dominant negatives, this study provides no direct evidence for this role, allowing the possibility that these CS-RPTPs may be functioning via another dominant mechanism.

**CS-RPTP Expression Does Not Grossly Influence Cell Fate Determination or RGC Axon Guidance**

CRYP- $\alpha$ , PTP- $\delta$ , and LAR are expressed in the optic vesicle prior to retinal cell differentiation, and con-

**A.****B.**

**Figure 6** Axons expressing CS-PTP- $\delta$  show retarded growth *in vivo*. (A) Wholemount analysis at stage 39 of axon outgrowth from RGCs expressing CS-CRYP- $\alpha$ +GFP-myc, CS-PTP- $\delta$ +GFP-myc, CS-LAR+GFP-myc, and GFP-myc alone following stage 19 retinal lipofection of DNA. The positions of growth cones within the optic tract were grouped into one of six bins based on their position in the optic pathway (see GFP-myc panel). Scale bar = 100  $\mu$ m. (B) Statistical analysis (chi-squared tests) of the positions of growth cones expressing different CS-RPTP constructs demonstrates that axons expressing CS-PTP- $\delta$  were significantly shorter than axons expressing GFP-myc alone, while CS-CRYP- $\alpha$  and CS-LAR had no significant effect. \*\*\* $p < .01$ .

**Table 2**  $p$ -Values of  $\chi^2$  Tests Comparing the Positions of Growth Cones Following Wholemount Analysis of DNA Lipofected Embryos

$\chi^2$ Test vs. GFP-myc:	Stage 35/36	Stage 37/38	Stage 39	Stage 40
CS-CRYP- $\alpha$	.338	.185	.511	.124
CS-PTP- $\delta$	.647	.107	.008	.052
CS-LAR	.575	.109	.275	.109

The positions of growth cones expressing each CS-RPTP construct at each stage were compared to the positions of GFP-myc expressing growth cones at a corresponding stage.

tinue to be expressed by subsets of cells in the retina until at least stage 41 (Johnson and Holt, 2000). Previous studies have demonstrated that numerous RPTKs are involved in *Xenopus* retinal cell fate determination; expression of a dominant negative FGFR decreases the percentage of photoreceptors by 50%, and generates a 350% increase in the percentage of amacrine cells (McFarlane et al., 1998), while expression of constitutively active c-src, a cytoplasmic tyrosine kinase, or its neural splice variant n-src, can specifically inhibit photoreceptor cell fate determination, decreasing the percentage of photoreceptors by 72% (Worley et al., 1997). Thus, it appears that tyrosine phosphorylation is an important mediator of cell fate determination in the *Xenopus* retina. However, overexpression of CS-CRYP- $\alpha$ , CS-PTP- $\delta$ , or CS-LAR, either individually or together, had no dramatic effect on cell fate determination in the *Xenopus* retina. This suggests that either the cell fate changes caused by overexpressing the CS-RPTPs were too subtle to be detected, or that CS-RPTP expression does not affect retinal cell fate determination.

RGC axons expressing CS-CRYP- $\alpha$ , CS-PTP- $\delta$ , or CS-LAR also did not exhibit severe axon guidance defects *in vivo*. Analysis of sectioned embryos showed that RGC axons expressing any one of these putative dominant constructs were able to grow out of the retina, cross at the chiasm, and extend up the optic tract to the contralateral tectum. Wholemount analysis of lipofected embryos, or of embryos that received a retinal transplant expressing CS-CRYP- $\alpha$ , CS-PTP- $\delta$ , CS-LAR, or CS-H3P, demonstrated that RGC axons expressing CS-RPTPs do not exhibit any major defects in axon guidance from the retina to the tectum. Axons are able to grow along the lateral surface of the diencephalon, turn posteriorly in the midoptic tract, enter the tectum, and terminate growth. It appears that RGCs expressing these CS-RPTPs are able to recognize all the major cues all along the optic pathway and interpret them appropriately. However, more subtle changes, such as altered fasciculation, improper optic

nerve fiber organization, or defects in terminal branching may be present that were not detected in this study.

Topographic mapping of retinal axons along the A-P axis of the optic tectum, in many species, involves the regulated activity of the EphA family of tyrosine kinases (Cheng et al., 1995; Drescher et al., 1995; Ernst et al., 1998; Frisen et al., 1998; Hornberger et al., 1999; Nakamura et al., 1994). If the type IIa RPTPs antagonize the EphAs, overexpression of putative dominant negative RPTP should result in a net increase in EphA tyrosine kinase activity. Overexpression of EphAs has been shown to cause axons to stop at more anterior positions in the tectum (Brown et al., 2000). Therefore, if these RPTPs negatively regulate the EphAs, the phenotype observed following overexpression of a dominant negative RPTP would be expected to be similar to the phenotype observed following overexpression of an active EphA receptor. Although CS-RPTP-expressing axons appear to grow normally and do not terminate exclusively at the anterior border of the tectum, as might be predicted by activating EphAs, more subtle defects in topographic mapping may be present that were not detected in this study. Because anterior-posterior topography is not easily detectable in *Xenopus* until a later stage than was addressed in this study (due to poor CS-RPTP expression at late stages), the effects on anterior-posterior map formation within the tectum could not be directly addressed. The use of a system more amenable to the analysis of tectal topography could provide further insight into the possible roles of these RPTPs in topographic map formation.

There are several other events in the formation of the retinotectal connection that were not addressed in this study. The fiber reorganizations that occur at the optic nerve behind the retina (Taylor, 1987), at the chiasm (Fawcett et al., 1984), and in the optic tract (Fawcett and Gaze, 1982) were not examined following the overexpression of CS-RPTPs. Additionally, terminal branching in the tectum and synapse formation were not examined.

**Table 3**  $p$ -Values of  $\chi^2$  Tests Comparing the Positions of Growth Cones at Stage 39 Following Wholemount Analysis of DNA Lipofected Embryos

	CS-CRYP- $\alpha$	CS-PTP- $\delta$	CS-LAR
$\chi^2$ Test vs. GFP-myc	.69	.0027	.96
$\chi^2$ Test vs. all other constructs	.50	.0004	.18

The positions of growth cones expressing each CS-RPTP construct were grouped into either the ventral (bin 1–3) or dorsal (bin 4–6) half of the optic pathway.  $p$ -values of  $\chi^2$  analysis of  $2 \times 2$  tables are shown.



**Table 4** Average Lengths of CS-RPTP-Expressing Neurites Grown on Basement Membranes with and without Glial Endfeet

Construct(s) Expressed	Mean Neurite Length on BM + EF (in $\mu M$ )	Mean Neurite Length on BM-EF (in $\mu M$ )
GFP-myc	131.1 ( $n = 102$ )	189.1 ( $n = 191$ )
CS-CRYP- $\alpha$ +GFP-myc	199.5*** ( $n = 182$ )	186.7 ( $n = 60$ )
CS-PTP- $\delta$ +GFP-myc	109.6* ( $n = 78$ )	196.5 ( $n = 104$ )
CS-LAR+GFP-myc	131.2 ( $n = 276$ )	177.6 ( $n = 168$ )
CS-H3P+GFP-myc	123.8 ( $n = 188$ )	183.9 ( $n = 147$ )

\*\*\*  $p < 0.001$ , \*  $p < .05$ .

Thus, although CS-RPTP expression has not demonstrated an obvious role for these RPTPs in regulating RGC axon guidance, they may be involved in the formation of the retinotectal system in ways that were not addressed in this study, or in ways too subtle to be detected using this analysis.

### CS-RPTP Expression Differentially Influences RGC Axon Outgrowth *in Vivo* and *in Vitro*

Overexpression of CS-PTP- $\delta$ , or CS-H3P in RGCs results in a significant inhibition of RGC axon outgrowth. At stage 39, 39% of RGC axons expressing GFP-myc alone, but only 25% of RGCs expressing PTP- $\delta$ , reached the tectum [Fig. 6(B)]. At stage 40, 77% of GFP-myc-expressing RGC axons, but only 61% of CS-H3P-expressing axons reached the tectum. Statistical analysis comparing the positions of growth cones expressing CS-PTP- $\delta$  and CS-H3P with GFP-myc, showed that there was a significant difference in the distribution of these growth cones in the optic tract ( $p < .01$ ). While CS-LAR- and CS-CRYP- $\alpha$ -expressing RGC growth cones were also more frequently found short of the tectum than GFP-myc-expressing RGC growth cones, statistical analysis did not indicate with confidence that these constructs significantly altered RGC axon outgrowth *in vivo* ( $p = .28$  and  $.51$ , respectively).

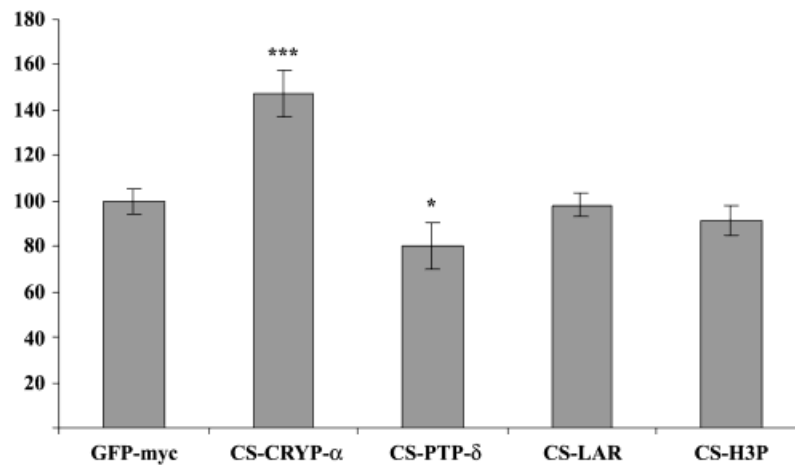
The decrease in axon length caused by the overexpression of CS-PTP- $\delta$  or CS-H3P suggests three possibilities: first, that RGCs expressing these constructs grow more slowly *in vivo*; second, that RGCs expressing these constructs are delayed in axonogenesis; or third, that these axons stall in the optic tract. *In vivo* time-lapse imaging of live RGC growth cones would best address this issue, but data gathered in these studies may also provide some insight. If RGCs were delayed in differentiation, one would expect this delay to be detectable at all stages, while if RGCs were growing more slowly or were stalled in the optic

tract, the delay would be more apparent at later stages of development, as the difference in length between normal axons and CS-RPTP-expressing axons increased. Analysis of RGC axons expressing these CS-RPTP constructs at multiple stages indicated that at stages 35/36 and 37/38 there were no significant differences between the positions of RGC axons in the optic tract, but this difference became significant as developmental time proceeded. This lack of statistical significance at earlier stages, however, may also be due to the fact that fewer axons had entered the optic tract at these younger stages, and therefore fewer axons were measured.

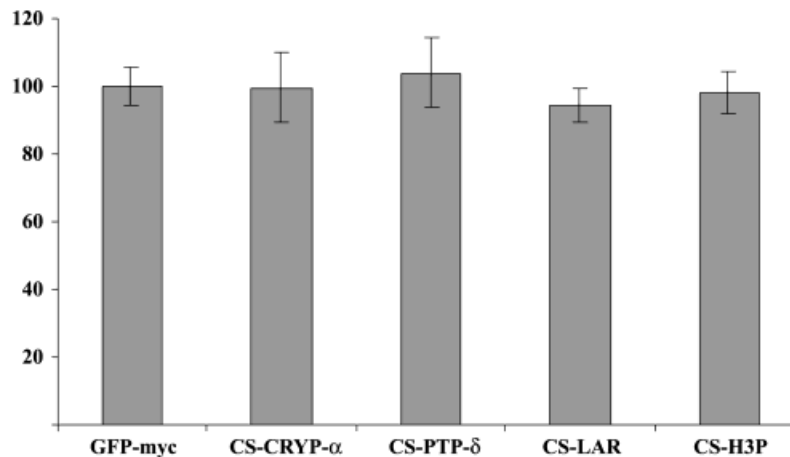
The first RGCs axons are initiated at stage 27/28 (Cima and Grant, 1980; Holt, 1984). These axons grow toward the optic nerve head and begin to enter the optic nerve at stage 29/30 (Cima and Grant, 1980; Holt, 1984). Wholemount retinas expressing these CS-RPTP constructs following a RNA blastomere injection did not show a dramatic difference in process initiation or outgrowth at stage 31, as numerous CS-RPTP-expressing RGC axons were seen entering the optic nerve head at stage 31 (data not shown). The presence of RGC axons at the optic nerve head of these wholemount retinas, and the normal pattern of intraretinal axon outgrowth in retinas expressing either the CS-RPTP constructs or GFP-myc, suggests that overexpression of CS-RPTP constructs does not significantly delay RGC differentiation or axonogenesis. It is more likely that the shorter axons in the brain are a result of a reduction in the rate of axon outgrowth, or the presence of stalled RGC axons in the optic tract.

PTP- $\delta$  is a homophilic neurite outgrowth-promoting cell adhesion molecule (Wang and Bixby, 1999). Previous studies have suggested that PTP- $\delta$  is expressed by retinal Mueller glia in the chick (Shock et al., 1995) and by retinal ganglion cells and the majority of inner nuclear layer cells (likely including Mueller glia) in *Xenopus* (Johnson and Holt, 2000). *In vivo*, expression of CS-PTP- $\delta$  reduces RGC axon

## A. Normalized Growth on Basement Membranes with Glial Endfeet



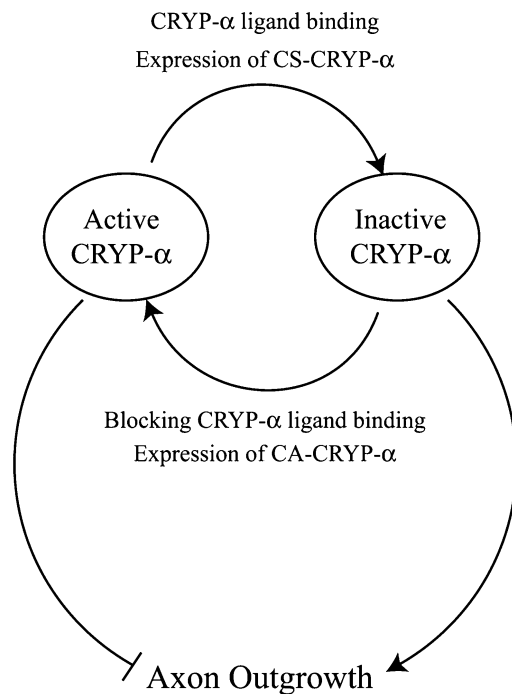
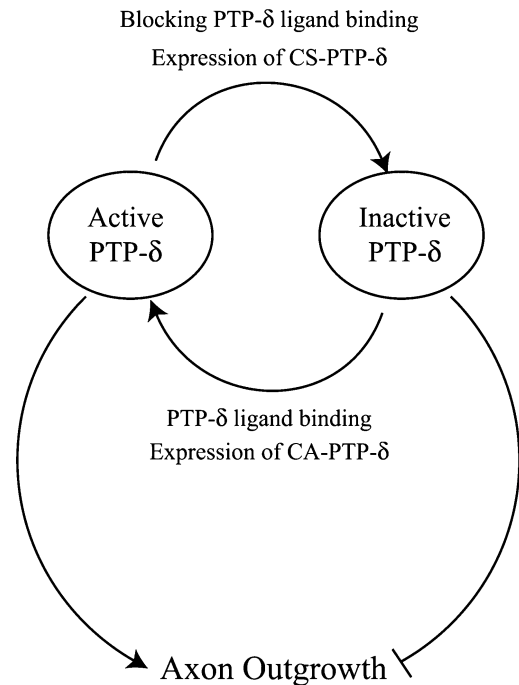
## B. Normalized Growth on Basement Membranes without Glial Endfeet



**Figure 7** Differential effects of CS-RPTP expression on retinal neurite extension *in vitro*. Analysis of normalized neurite outgrowth on basement membranes with and without glial endfeet. (A) Retinas from DNA blastomere injected embryos expressing GFP-myc, CS-CRYP-α+GFP-myc, CS-PTP-δ+GFP-myc, CS-LAR+GFP-myc, or CS-H3P+GFP-myc were cultured for 20 h on retinal basement membranes isolated from E7 chick retinas. CS-CRYP-α expression significantly increases, while CS-PTP-δ expression significantly decreases, the average neurite length. (B) On E7 chick retinal basement membranes without glial endfeet there is no significant difference in neurite length between cells expressing any or all of the CS-RPTP constructs. CS-H3P, coexpression of all three CS-RPTP constructs.

length by approximately 18%. A similar reduction in the rate of axon outgrowth was also seen following CS-PTP-δ expression on basement membranes with glial endfeet, but not on basement membranes without glial endfeet. While the removal of glial endfeet has been demonstrated to remove the majority of the CRYP-α ligand (Ledig et al., 1999a), and is hypothesized to remove the PTP-δ expressed by glial endfeet, this detergent treatment will also remove a

variety of other unrelated molecules. Thus, axon outgrowth on basement membranes without glial endfeet will be affected not only by removal of putative RPTP ligands, but also by the removal of any other molecules that wash off in detergent. However, by comparing GFP-myc-expressing RGCs with CS-PTP-δ-expressing RGCs on this substrate, one can get an indication of whether the effect of CS-PTP-δ expression is dependent on the presence of a molecule

A. Proposed model for CRYP- $\alpha$ B. Proposed model for PTP- $\delta$ 

**Figure 8** Two models illustrating potential mechanisms for CRYP- $\alpha$  and PTP- $\delta$  regulation of *Xenopus* RGC axon outgrowth, based upon the hypothesis that the CS-RPTPs function as dominant negative constructs. (A) Blocking CRYP- $\alpha$  ligand-receptor interactions decreases axon outgrowth (Ledig et al., 1999a), while expression of CS-CRYP- $\alpha$  increases axon outgrowth on retinal basement membranes. This suggests that CRYP- $\alpha$  ligand binding functions in the same direction as CS-CRYP- $\alpha$  expression. Operating under the hypothesis that CS-CRYP- $\alpha$  functions as a dominant negative, this suggests that CRYP- $\alpha$  ligand binding and CS-CRYP- $\alpha$  expression both inactivate CRYP- $\alpha$  to promote axon outgrowth, while blocking CRYP- $\alpha$  ligand binding or expressing a constitutively active (CA) CRYP- $\alpha$  might be hypothesized to inhibit axon outgrowth. (B) PTP- $\delta$  is a homophilic cell adhesion molecule that increases the rate of axon outgrowth (Wang and Bixby, 1999) through homophilic interactions, while expression of CS-PTP- $\delta$  inhibits RGC axon outgrowth. This suggests that PTP- $\delta$  receptor-ligand interactions function in the opposite direction as CS-PTP- $\delta$  expression. If CS-PTP- $\delta$  functions as a dominant negative, this suggests that PTP- $\delta$  homophilic interactions activate PTP- $\delta$  to promote axon outgrowth, while CS-PTP- $\delta$  expression inactivates PTP- $\delta$  to inhibit axon outgrowth. No effect of CS-LAR expression was observed on RGC axon outgrowth.

associated with the glial endfeet, a likely candidate being PTP- $\delta$  itself.

The observed reduction in neurite outgrowth caused by overexpression of CS-PTP- $\delta$  may be a result of blocking PTP- $\delta$  signaling following the homophilic binding of PTP- $\delta$  molecules on RGCs and glial endfeet. Homophilic binding of PTP- $\delta$  enhances neurite outgrowth (Wang and Bixby, 1999), while expressing CS-PTP- $\delta$  inhibits outgrowth. This suggests that homophilic PTP- $\delta$  interactions may activate

the phosphatase activity of PTP- $\delta$ , and enhance RGC axon outgrowth (Fig. 8). This model could be more rigorously tested by pre-incubating basement membranes with a PTP- $\delta$  antibody and analyzing RGC axon outgrowth on this treated substrate in the presence and absence of CS-PTP- $\delta$ , or by analyzing the effects of CS-PTP- $\delta$  expression on axon outgrowth on a PTP- $\delta$  substrate.

Overexpression of the CS-CRYP- $\alpha$  construct significantly enhances neurite outgrowth on basement

membranes with glial endfeet, but shows no effect on outgrowth in the absence of glial endfeet. Because a ligand for CRYP- $\alpha$  is expressed predominantly on retinal Mueller glial endfeet in chick (Ledig et al., 1999a) and in *Xenopus* (data not shown), the effects of CS-CRYP- $\alpha$  overexpression appear to be dependent on the presence of a CRYP- $\alpha$  ligand. In the presence of glial endfeet, GFP-myc-expressing neurites grow 30% slower than in the absence of glial endfeet, confirming the observation that glial endfeet have a net inhibitory effect on neurite elongation (Ledig et al., 1999a). The outgrowth inhibition caused by glial endfeet is completely abolished by overexpressing CS-CRYP- $\alpha$ , suggesting that the growth inhibitory cues on glial endfeet may signal through the CRYP- $\alpha$  receptor. Because inhibiting CRYP- $\alpha$  function (by expressing a CS-CRYP- $\alpha$  construct) has the same effect on neurite outgrowth as removing CRYP- $\alpha$  ligands (by removing the glial endfeet), it seems logical that in the absence of these CRYP- $\alpha$  ligands, the receptor has a reduced level of activity and that the ligand binding to CRYP- $\alpha$  normally activates the CRYP- $\alpha$  receptor. This suggests that the CRYP- $\alpha$  ligand is a growth inhibitory molecule that binds to and activates the CRYP- $\alpha$  receptor. The presence of this repulsive molecule contributes to the inhibitory effect that glial endfeet have on neurite outgrowth, an inhibition that is abolished by blocking CRYP- $\alpha$  activity.

While the above is an appealing hypothesis for CRYP- $\alpha$  function, Ledig et al. (1999a) previously demonstrated that blocking CRYP- $\alpha$  ligand-receptor interactions, either at the ligand or at the receptor level, significantly reduces the length of RGC axons on basement membranes with glial endfeet. This inhibition of outgrowth caused by blocking ligand-receptor interactions only occurs in the presence of high levels of the CRYP- $\alpha$  ligand (i.e., only on basement membranes with glial endfeet) (Ledig et al., 1999a). Their data demonstrate that the CRYP- $\alpha$  ligand is an outgrowth promoting molecule, exactly the opposite prediction made based on the data in this study.

There is an alternative hypothesis, however, that explains the fact that overexpression of CS-CRYP- $\alpha$  enhances neurite outgrowth, while blocking ligand binding inhibits axon outgrowth. We have shown that the overexpression of a putative dominant negative CRYP- $\alpha$  increases process outgrowth, an opposite effect to that seen by blocking ligand binding (Ledig et al., 1999a). Thus, receptor activation and ligand binding would appear to signal in opposite directions. These data suggest a hypothesis for CRYP- $\alpha$  function based on ligand-induced receptor inactivation. This hypothesis proposes that CRYP- $\alpha$ -ligand binding

turns off the CRYP- $\alpha$  receptor and enhances outgrowth, and assumes that in the absence of ligand, some fraction of the endogenous receptor is active. Ligand binding to CRYP- $\alpha$  causes the inactivation of a subset of the active receptor pool, resulting in a net promotion of axon outgrowth. Similarly, overexpression of CS-CRYP- $\alpha$  blocks the activity of the active receptor pool, also promoting neurite outgrowth (Fig. 8). This model is consistent with the previous studies by Ledig et al., as well as with the data obtained in this study.

There is also a biological precedent for this hypothesis. Pleiotrophin is the natural ligand of PTP- $\zeta$ , a RPTP that binds to and dephosphorylates  $\beta$ -catenin (Meng et al., 2000). Pleiotrophin/PTP- $\zeta$  interactions turn off the catalytic activity of PTP- $\zeta$  *in vitro* and *in vivo*, resulting in a net increase in the level of tyrosine phosphorylation on  $\beta$ -catenin (Meng et al., 2000). Thus, CRYP- $\alpha$  receptor/ligand interactions may function in a similar way to PTP- $\zeta$  receptor/ligand interactions, where ligand binding induces the inactivation of the cytoplasmic phosphatase domains. To further test this hypothesis, it would be informative to investigate how a constitutively active CRYP- $\alpha$  receptor would function to regulate neurite outgrowth.

If CS-CRYP- $\alpha$  expression blocks the function of the endogenous CRYP- $\alpha$  and promotes axon outgrowth, why then when all three CS-RPTP constructs are expressed is there no net increase in axon length? Expression of CS-PTP- $\delta$  inhibits axon outgrowth, while expression of CS-CRYP- $\alpha$  promotes axon outgrowth. One might expect that expressing both of these constructs together would result in an intermediate axon length, a balance between blocking the outgrowth promoting activities of PTP- $\delta$  activity and the outgrowth inhibiting effects of CRYP- $\alpha$  activity. This is indeed the case, as overexpression of CS-H3P does not significantly increase or decrease mean neurite length when compared to GFP-myc alone. This suggests that on retinal basement membranes, the growth cone senses and responds to both growth promoting and growth inhibiting cues through PTP- $\delta$  and CRYP- $\alpha$ , respectively.

If the ligand-induced receptor inactivation hypothesis is correct, overexpression of CS-CRYP- $\alpha$  should enhance outgrowth independent of the CRYP- $\alpha$  ligand. On basement membranes without glial endfeet, overexpression of CS-CRYP- $\alpha$  does not, in fact, exhibit such an effect. However, we view this substrate as one of the most potent neurite outgrowth promoters, and it is quite likely that the RGC axon is saturated with other outgrowth promoting cues. It seems possible that the loss of CRYP- $\alpha$  activity, which would normally inhibit the rate of outgrowth, might



have little effect on a system inundated with outgrowth promoting cues, but may have more of an effect in an environment with a balance of outgrowth promoting and outgrowth inhibiting cues.

On a relatively simple substrate like laminin, there should be no ligands for these RPTPs, as the LAR expressed in *Xenopus* (Johnson and Holt, 2000) lacks the laminin-nidogen complex binding motif (O'Grady et al., 1998) and the substrate specificity of CS-RPTP action could, in theory, be tested. However, the LAR family of RPTPs share secondary signaling proteins with the integrins; the regulation of outgrowth promotion in both cases appears to be mediated by Rac (Bateman et al., 2000; Schoenwaelder and Burridge, 1999). Thus, one might expect that the overexpression of CS-RPTPs would result in the sequestration of Rac, causing an inhibition of integrin-mediated outgrowth. Indeed, expression of any or all of the CS-RPTPs caused a significant decrease in RGC axon length on laminin (data not shown). Unfortunately, the interpretation of these results is complicated by the fact that either competition for shared signaling proteins, or a direct inhibition of the endogenous RPTP could cause such a growth retardation. The identification of ligands and substrates for these RPTPs will greatly enhance the ability to interpret such data.

### Comparison of *in Vitro* and *in Vivo* Data

CS-PTP- $\delta$  expression is a potent inhibitor of neurite outgrowth both *in vivo* and *in vitro*, while CS-CRYP- $\alpha$  expression enhances neurite outgrowth *in vitro* but has no effect *in vivo*. How can the disparity between the *in vitro* and the *in vivo* effects of CS-CRYP- $\alpha$  expression be explained? The *in vivo* substrate is much more complex than the *in vitro* substrate. *In vivo*, RGC axons do not grow along a uniform substrate from the retina to the tectum, but instead navigate along the retinal basement membrane, dive through the neural retina at the optic nerve head, traverse along the basement membrane surrounding the optic nerve, cross at the chiasm, and grow up the contralateral optic tract to the tectum. These diverse substrates express complex patterns of neurite outgrowth promoting and neurite outgrowth inhibiting molecules. Certainly, CS-RPTP expression affects neurite outgrowth on retinal basement membranes. However, in other locations along the optic pathway, overexpressing CS-CRYP- $\alpha$  may also affect neurite outgrowth. CS-CRYP- $\alpha$ -expressing RGCs may grow faster along the retinal basement membrane and slow down once they leave this substrate, resulting in no net change in the overall rate of axon outgrowth. Future studies using time-lapse video mi-

croscopy may help formulate more accurate models for how these RPTPs regulate axon outgrowth at different parts of the optic pathway *in vivo*.

One certainty that emerges is that the analysis of effects caused by overexpressing these CS-RPTP constructs is complex. CS-RPTPs may function as dominant negatives, mediating their effects through a direct inhibition of the endogenous receptor. The models proposed in this article (Fig. 8) regarding the interactions between CRYP- $\alpha$  and PTP- $\delta$  activity and axon outgrowth are designed based on the results obtained by examining the outgrowth of neurites expressing these constructs on basement membranes with glial endfeet, and therefore, best reflect the role of these RPTPs in intraretinal axon outgrowth. At other places in the optic pathway, these models may need to be modified to more accurately reflect the molecular environment in which the endogenous protein is functioning. Nevertheless, they do illustrate testable hypotheses for how these RPTPs function in RGC axon outgrowth in the vertebrate visual system.

The authors would like to thank Suchita Shah for her detailed analysis of putative CRYP- $\alpha$  ligand expression in the developing *Xenopus* visual system, and John Steele for his invaluable help with statistics.

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