

# CRYP-2/cPTPRO is a neurite inhibitory repulsive guidance cue for retinal neurons in vitro

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Receptor protein tyrosine phosphatases (RPTPs) are implicated as regulators of axon growth and guidance. Genetic deletions in the fly have shown that type III RPTPs are important in axon pathfinding, but nothing is known about their function on a cellular level. Previous experiments in our lab have identified a type III RPTP, CRYP-2/cPTPRO, specifically expressed during the period of axon outgrowth in the chick brain; cPTPRO is expressed in the axons and growth cones of retinal and tectal projection neurons. We constructed a fusion protein containing the extracellular domain of cPTPRO fused to the Fc portion of mouse immunoglobulin G-1, and used it

to perform in vitro functional assays. We found that the extracellular domain of cPTPRO is an antiadhesive, neurite inhibitory molecule for retinal neurons. In addition, cPTPRO had potent growth cone collapsing activity in vitro, and locally applied gradients of cPTPRO repelled growing retinal axons. This chemorepulsive effect could be regulated by the level of cGMP in the growth cone. Immunohistochemical examination of the retina indicated that cPTPRO has at least one heterophilic binding partner in the retina. Taken together, our results indicate that cPTPRO may act as a guidance cue for retinal ganglion cells during vertebrate development.

## Introduction

Axons are guided to their targets by a complex complement of extracellular navigational cues. These cues may be diffusible or substrate-bound molecules, but each interacts with specific neuronal receptors, initiating signaling cascades within the neuron that ultimately regulate the assembly of cytoskeletal elements and the addition of axonal membrane. The regulatory ligands influencing axon growth and guidance are a diverse lot, including cell adhesion molecules (CAMs),\* netrins, semaphorins, slits, and ephrins (Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Drescher et al., 1997; Harris and Holt, 1999). These proteins are linked together in that their signaling depends on tyrosine phosphorylation: either their receptors are tyrosine kinases

or they regulate signaling pathways that are dependent on tyrosine kinases (Walsh and Doherty, 1996; Ming et al., 1999; Tamagnone et al., 1999). Thus, the enzymes controlling tyrosine phosphorylation are critically important in axon growth and guidance. In particular, the key roles played by receptor protein tyrosine phosphatases (RPTPs) in axon growth and guidance are increasingly appreciated (for review see Bixby, 2000). The most compelling evidence for such roles comes from genetic studies in *Drosophila*, in which loss-of-function mutations in CAM-like type IIa (Dlar, PTP69D) and type III (PTP99A, PTP10D) RPTPs cause severe neuronal pathfinding defects (Desai et al., 1996; Krueger et al., 1996; Sun et al., 2000a, 2001).

In vertebrates, there is substantial evidence that type II RPTPs influence axon growth and guidance. PTP- $\sigma$  (CRYP- $\alpha$  in the chick), a type IIa RPTP, promotes retinal ganglion cell (RGC) outgrowth on basement membranes (Ledig et al., 1999a). PTP- $\delta$  is a homophilic CAM that promotes outgrowth of forebrain neurons (Wang and Bixby, 1999) and is an attractive guidance cue in vitro (Sun et al., 2000b). PTP- $\sigma$  and leukocyte antigen-related protein (LAR) knockout mice have specific neurological defects, some of which relate to axon growth (Yeo et al., 1997; Elchebly et al., 1999; Wallace et al., 1999). As for the type IIb RPTPs, both PTP- $\mu$  and PTP- $\kappa$  promote neurite outgrowth from cultured primary neurons (Burden-Gulley and Brady-Kal-

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\*Abbreviations used in this paper: CAM, cell adhesion molecule; ECD, extracellular domain; LN, laminin; PDL, poly-D-lysine; PTP, protein tyrosine phosphatase; RGC, retinal ganglion cell; RPTP, receptor PTP.

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may, 1999; Drosopoulos et al., 1999). In contrast, essentially nothing is known about the function of type III RPTPs in the vertebrate nervous system. As mentioned above, experiments in *Drosophila* suggest that the type III RPTPs PTP99A and PTP10D are involved in axon pathfinding. Although null mutants of these RPTPs have no discernible phenotype, mutations in either of these molecules significantly alter the neural phenotypes of type IIa mutants (Desai et al., 1997; Sun et al., 2000a). However, these studies have not provided information on the mechanisms underlying RPTP function.

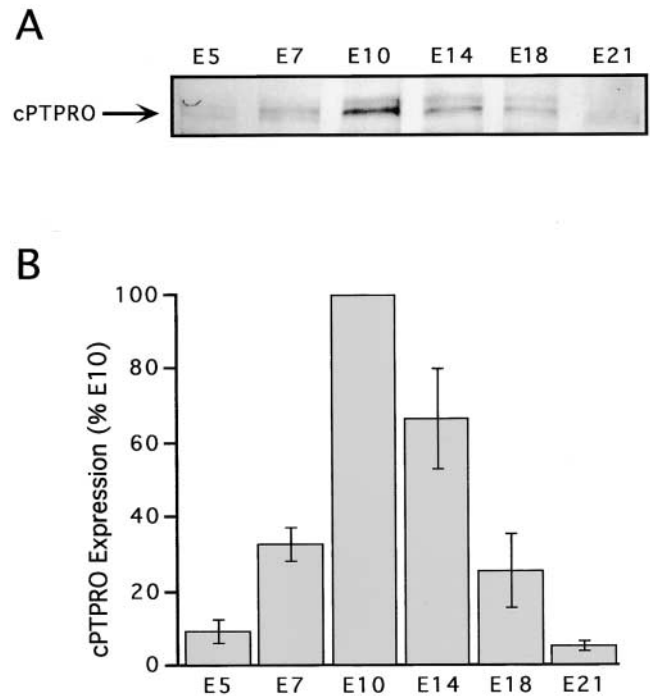
In a PCR-based screen of the chick brain, our lab identified a novel type III RPTP that we named CRYP-2 (Bodden and Bixby, 1996). Primary amino acid sequence analysis indicates that CRYP-2 is a transmembrane molecule with eight extracellular fibronectin type III repeats and one cytoplasmic phosphatase domain. Outside of the nervous system, expression is seen only in the kidney (Chilton and Stoker, 2000). Homologues of CRYP-2 have been found in rabbits (GLEPP-1; Thomas et al., 1994), rats (RPTP-BK; Tagawa et al., 1997), mice (mGLEPP/mPTPRO; Tomemori et al., 2000; Wang et al., 2000), and humans (Seimiya et al., 1995; Wiggins et al., 1995). We believe that these proteins all represent orthologues (unpublished data), and we will henceforth use cPTPRO to denote the chick isoform.

PTPRO is expressed at the right time and in the right place to be important in axon growth and guidance. PTPRO mRNA is selectively expressed in neurons in the brain during the time of axon outgrowth in the chick embryo (Bodden and Bixby, 1996). In the retina, cPTPRO is selectively expressed in the projection neurons (RGCs) and is concentrated in axons and growth cones. PTPRO is also expressed in the optic tectum, the major target of the retinal projection (Ledig et al., 1999b). This pattern of expression, together with the genetic evidence involving cPTPRO's closest relative in the fly, PTP10D, suggests that cPTPRO plays a role in axon growth and guidance. In this study, we used a variety of *in vitro* assays to investigate the potential functions of cPTPRO. Our results indicate that in contrast to most other RPTPs examined, the cPTPRO extracellular domain (ECD) is antiadhesive and neurite inhibitory, and acts as a repulsive guidance cue for retinal neurons.

## Results

### cPTPRO protein is developmentally regulated in embryonic chick brain

cPTPRO mRNA is upregulated between embryonic days 8 (E8) and 13 (E13) in chick brain, coinciding with the major period of axon outgrowth (Bodden and Bixby, 1996). To determine if cPTPRO protein expression follows this pattern, we used an antibody to a fusion protein from the ECD of cPTPRO (Ledig et al., 1999b) to probe Western blots of membrane fractions from chick brain. This antibody recognized a specific cPTPRO band at 180 kD that was not present in membranes from liver or muscle (unpublished data). This size is similar to that seen for cPTPRO orthologues in other species (Tagawa et al., 1994; Thomas et al., 1994; Wiggins et al., 1995), and is the size encoded by the



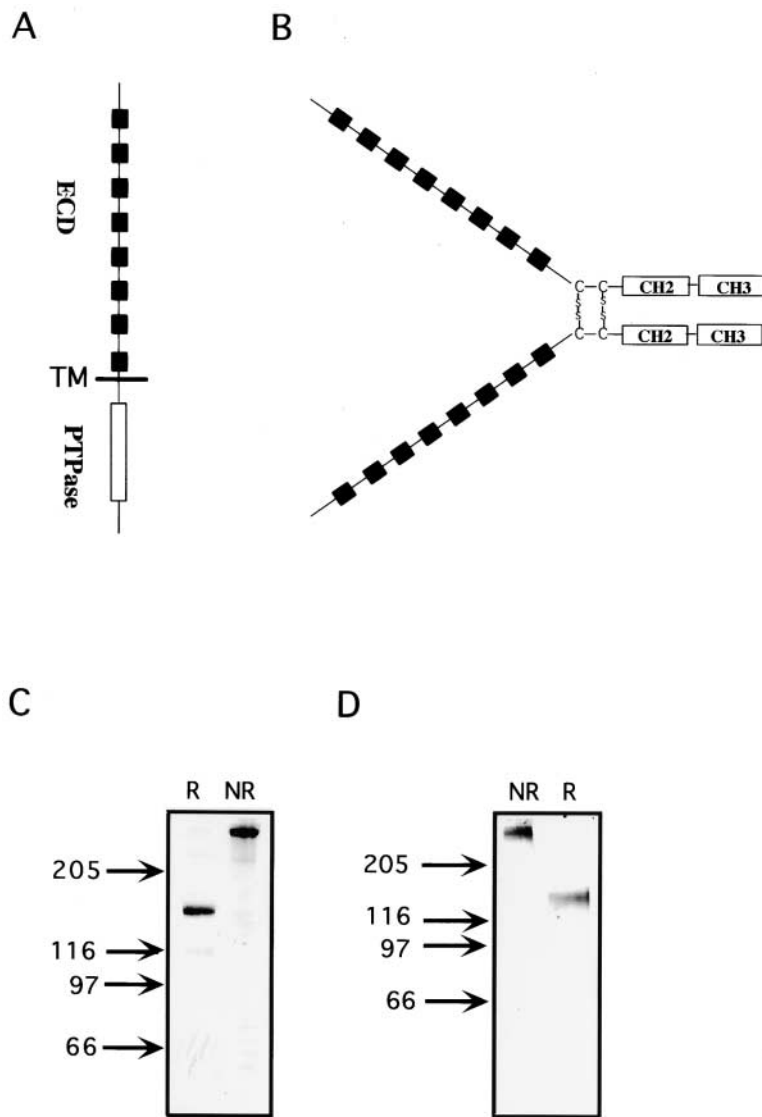
**Figure 1. cPTPRO protein is developmentally regulated in embryonic chick brain.** 20  $\mu$ g of brain P2 fractions from E5, E7, E10, E14, E18, and E21 was separated by SDS-PAGE and subjected to Western blot analysis using affinity-purified anti-cPTPRO antibody. (A) cPTPRO migrated with an apparent molecular mass of 180 kD (arrow). Note the increase in expression from E5 to E10 and the decrease after E14. (B) Quantitative analysis from three independent experiments. Data were plotted as a percentage of the levels at E10  $\pm$  SEM.

full-length cDNA when expressed in transfected COS cells (unpublished data). As the predicted protein encoded by the PTPRO cDNA is 141 kD (Bodden and Bixby, 1996), our data are consistent with a high degree of glycosylation. PTPRO expression in brain was low at E5, increased dramatically between E7 and E10, then declined after E14, consistent with our previous findings for mRNA expression (Fig. 1). This pattern is similar to that suggested by immunofluorescence experiments in retina and tectum, and is consistent with our hypothesis that cPTPRO plays a role in axon outgrowth during development.

In an earlier study, we reported a band at 330 kD that was specifically recognized by an antibody to a cPTPRO peptide sequence (Bodden and Bixby, 1996). This band was sometimes faintly visible in Western blots with the fusion protein antibody (unpublished data). The relationship between this band and the 180-kD band is not known.

### Localization of cPTPRO binding sites in the retina

Although nothing is known concerning ligand–receptor interactions of type III RPTPs, several type II RPTPs can bind homophilically, serving as both ligands and receptors (Bixby, 2000). Ligand–receptor interactions of these CAM-like RPTPs as well as those of other CAMs have been examined productively using Fc fusion proteins of the ECDs (Walsh and Doherty, 1997; Drosopoulos et al., 1999; Wang and Bixby, 1999). To investigate these interactions for cPTPRO, we fused the cDNA encoding the ECD of



**Figure 2. Expression and purification of the cPTPRO-Fc fusion protein.** (A) Schematic diagram of cPTPRO. The horizontal line represents the plasma membrane (TM), with extracellular area above. The ECD of cPTPRO consists of eight fibronectin type III repeats (■). The intracellular portion contains one phosphatase domain (□). (B) Diagram of the fusion protein. The protein is a disulfide-linked (S-S) dimer, with the ECD of cPTPRO fused to the hinge and constant regions (CH2, CH3) of mouse IgG-1. (C) 1 µg of purified cPTPRO-Fc was run on SDS-PAGE and stained with Coomassie blue. (D) 20 µg of cPTPRO-Fc was subjected to Western blot analysis with an anti-cPTPRO antibody. cPTPRO-Fc migrated with an apparent molecular mass of 170 kD under reducing (R) conditions and 350 kD under nonreducing (NR) conditions. Numbered arrows represent the molecular mass in kD of molecular weight standards.

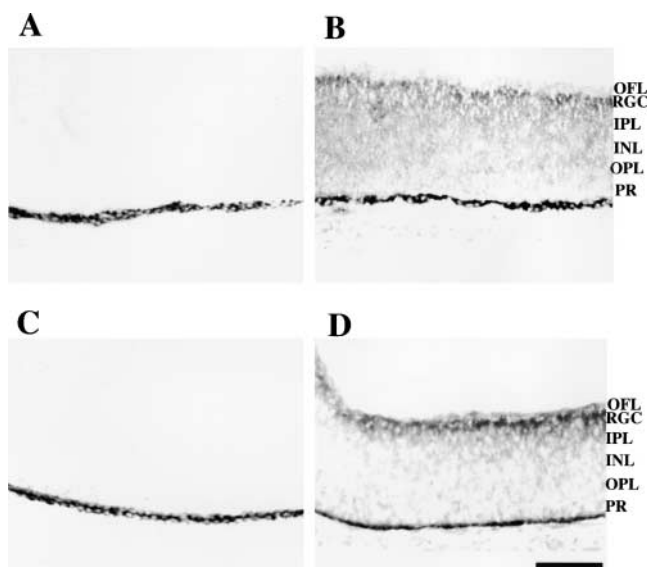
cPTPRO to the cDNA encoding the Fc domain of mouse IgG-1 (mIgG; Fig. 2, A and B). This construct was used to express the fusion protein (cPTPRO-Fc) in stably transfected CHO cells, and purified with anti-mIgG agarose. As expected, the purified protein migrated as a 170-kD band under reducing conditions and as a 350-kD dimer under nonreducing conditions (Fig. 2 C). Bands of these same sizes were identified by an anti-cPTPRO antibody on Western blots of the purified proteins (Fig. 2 D), confirming the presence of the cPTPRO ECD in the fusion protein.

Previous experiments demonstrated that cPTPRO is expressed in the embryonic chick retina. In particular, cPTPRO is strongly expressed in RGCs and their axons from E6 to E10 (when axons are growing to the tectum), in various fiber layers of the retina by E10, and becomes down-regulated after this time (Ledig et al., 1999b). We used the cPTPRO-Fc fusion protein to localize potential binding partners for cPTPRO in the retina. Sections of E7 chick retina were stained by binding cPTPRO-Fc to the sections, followed by anti-Fc immunohistochemistry to localize the bound fusion protein. cPTPRO binding sites were found in most of the neural retina (except the photoreceptor layer),

with the strongest staining in the RGC layer and the weakest in the outer plexiform layer (Fig. 3 B). The optic fiber layer, containing the axons of the RGCs, was nearly devoid of staining. Adjacent sections stained with antibodies to cPTPRO confirmed that the majority of cPTPRO immunoreactivity is found both in the optic fiber layer and the ganglion cell layer at this time (Fig. 3 D). Control sections stained with mIgG alone demonstrated the specificity of binding (Fig. 3, A and C). These results demonstrate the presence of at least one cPTPRO binding partner in the retina, which apparently is expressed in several cell types. Because the distribution of cPTPRO and its binding partner overlap but are not identical, our results suggest, but do not prove, that cPTPRO has at least one heterophilic binding partner in the retina during embryogenesis.

#### cPTPRO is antiadhesive for retinal neurons

The ECD of cPTPRO consists entirely of fibronectin type III repeats (Bodden and Bixby, 1996). Because these motifs are commonly found in the ECDs of CAMs (Baldwin et al., 1996), and in some cases appear to mediate cell adhesion (Frei et al., 1992; Appel et al., 1993), we predicted that the ECD of



**Figure 3. Localization of cPTPRO binding sites and cPTPRO in E7 chick retina.** 10- $\mu$ m sections of chick retina were cut on a cryostat. (A and B) 5  $\mu$ g/ml of cPTPRO-Fc was allowed to bind to E7 tissue sections (B). 10  $\mu$ g/ml of mIgG-1 was used as the negative control (A). Staining was most noticeable in the RGC layer, suggesting that this is the predominant location of binding partner(s) for cPTPRO. (C and D) Sections adjacent to those above were used to compare cPTPRO location. The optic fiber layer (OFL) and RGC layer were stained most strongly by anti-cPTPRO antibody (D). The IgG portion of preimmune serum was used as a control (C). Similar results were obtained in multiple experiments. INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; PR, photoreceptors. The dark stained layer in all panels is pigment epithelium. Bar, 100  $\mu$ m.

cPTPRO could be an adhesive substrate for neurons. To test this idea, we performed several types of cell adhesion assays. Initial adhesion experiments employed retinal neurons, as these appear to express a binding partner for the cPTPRO ECD. Surprisingly, few retinal neurons adhered to spots containing cPTPRO-Fc as a substrate, even though controls demonstrated that the neurons adhered to spots of known adhesive molecules (poly-D-lysine [PDL]) on the same dish. Similar results were obtained with forebrain, cerebellar, and ciliary ganglion neurons (unpublished data). These results suggest that the cPTPRO ECD does not support neuronal adhesion. However, because our ligand binding experiments suggest an interaction of the cPTPRO ECD with retinal neurons, we examined the possibility that cPTPRO-Fc could be an antiadhesive substrate. In these experiments, cPTPRO or mIgG as a control was spotted onto nitrocellulose substrates, and a larger spot of laminin (LN) was then placed over the first spot. This created a central spot of cPTPRO-Fc (or mIgG) mixed with LN, surrounded by a ring of LN alone. In our short-term assay, the center area containing LN plus cPTPRO-Fc did not support neuronal adhesion, though retinal neurons adhered well to the LN surround, or to the LN-mIgG mixture (Fig. 4 A). Indeed, neurons failed to adhere to or spread on the cPTPRO-LN mixture even after 24 h of culture (unpublished data). These results suggest that the cPTPRO ECD is antiadhesive for retinal neurons.

In the experiments described above, it is possible that cPTPRO-Fc somehow prevented LN from binding to the

substrate, rather than inhibiting adhesion. This seems unlikely, as LN binds strongly to nitrocellulose and higher concentrations of mIgG did not affect adhesion to LN. However, to test this idea we did experiments in which cPTPRO and LN were added simultaneously. We combined an optimal concentration of LN (20  $\mu$ g/ml) with varying concentrations of cPTPRO, and spotted the mixtures onto nitrocellulose-coated dishes. In these assays, concentrations of cPTPRO between 25 and 100  $\mu$ g/ml inhibit neuronal adhesion to LN in a dose-dependent manner, whereas mIgG had no effect on adhesion at 100  $\mu$ g/ml (Fig. 4 B). Thus, the inhibitory effect of cPTPRO-Fc is due to the cPTPRO ECD rather than the Fc fragment.

We also performed adhesion inhibition assays in which N-cadherin was used as the adhesive substrate. Similar to the situation for LN, cPTPRO-Fc inhibited retinal neuron adhesion to N-cadherin in a dose-dependent manner when the two were mixed before substrate coating (Fig. 4 C). Thus, the cPTPRO ECD prevents retinal neurons from adhering to substrates that are normally very adhesive. Because cPTPRO inhibits adhesion when spotted simultaneously with adhesive proteins, and because it inhibits adhesion mediated by two unrelated proteins, it is unlikely that inhibition is simply due to competition for binding sites on the substrate or on the adhering neurons, though this remains a possibility.

#### cPTPRO ECD inhibits retinal neurite outgrowth

Although nothing is known concerning the activities of type III RPTPs, the ECDs of several type II RPTPs have been shown to promote neurite growth in vitro (Burden-Gulley and Brady-Kalnay, 1999; Drosopoulos et al., 1999; Ledig et al., 1999a; Wang and Bixby, 1999). Furthermore, cPTPRO is localized on RGC axons and growth cones, putting it in the right location to influence axon growth (Ledig et al., 1999b). To test whether the ECD of cPTPRO can regulate neurite growth from retinal neurons, we compared the growth of retinal neurites on substrates of LN with growth on LN mixed with cPTPRO-Fc. As cPTPRO is antiadhesive, we first coated substrates with PDL to allow LN-independent adhesion of retinal neurons. Retinal neurons adhered well, but did not grow neurites when cultured overnight on PDL alone (Fig. 5 A). Neurons adhered and grew numerous processes on an LN-PDL substrate (Fig. 5 B). The presence of PDL allowed strong neuronal adhesion when cultures were grown on a cPTPRO-Fc substrate, but neurons mainly failed to extend neurites, showing that cPTPRO does not promote retinal neurite growth (unpublished data). More interestingly, LN-induced neurite formation was substantially inhibited by the presence of cPTPRO (Fig. 5, C and D). Quantification of neurite outgrowth confirmed that the presence of cPTPRO inhibited, in a dose-dependent manner, both the percentage of cells with neurites and the average length of individual neurites (see Fig. 7, A and B). In control experiments, mixing LN with IgG did not affect either parameter of neurite growth, even when the IgG was present at higher concentrations than the cPTPRO-Fc (see Fig. 7). To ensure that the inhibitory effect of cPTPRO was not due to competition for binding to the culture substrate, we performed experiments in which LN was spotted



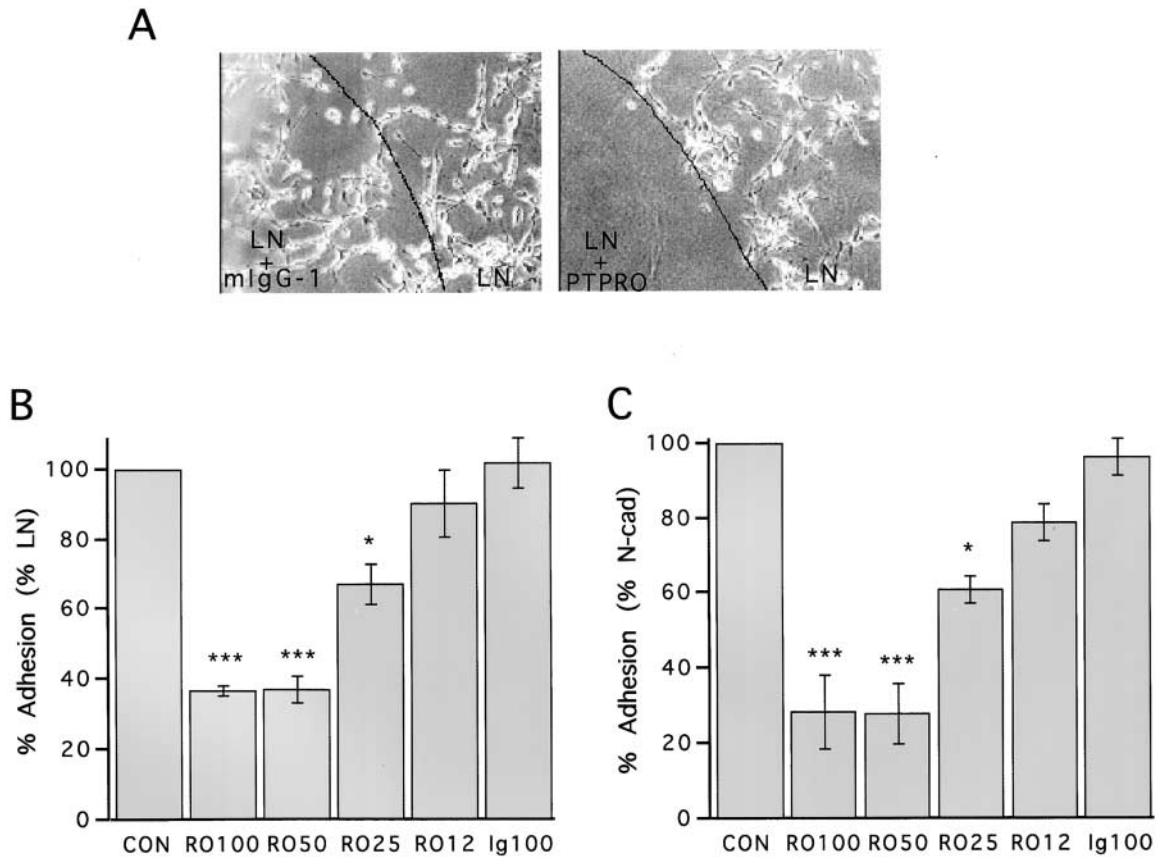


Figure 4. **cPTPRO is antiadhesive for retinal neurons.** Dissociated E6 retinal neurons were allowed to adhere for 2 h before fixation. (A) Neurons adhered equally well to the inner spot of LN/mIgG-1 and to the outer ring of LN alone (left). However, neurons did not adhere to an inner spot of LN/cPTPRO-Fc (right). (B) Quantitative data from adhesion assays in which cPTPRO-Fc or mIgG were mixed with LN and spotted onto nitrocellulose-coated dishes. Data are expressed as a percentage of neurons adhering to a spot of LN alone (CON) and plotted as mean  $\pm$  SEM for three separate experiments. A dose-dependent decrease in adhesion was apparent with increasing concentrations of cPTPRO, from 12 to 100  $\mu$ g/ml. No such decrease was caused by mIgG-1. (C) Similar results were obtained from three experiments in which cPTPRO-Fc or mIgG-1 were mixed with N-cadherin. Data were plotted as the percentage of neurons adhering to a spot of N-cadherin alone (CON). Again a dose-dependent decrease in adhesion was observed with increasing concentrations of cPTPRO-Fc.

onto the substrate before coating with cPTPRO. In two experiments, neurite outgrowth (measured as percentage of neurons with neurites) was inhibited 61% by PTPRO added to the substrate after the LN coating, compared with

69% inhibition by PTPRO added simultaneously with the LN. Taken together, these data indicate that the cPTPRO ECD can inhibit retinal neurite growth induced by substrate-bound LN.

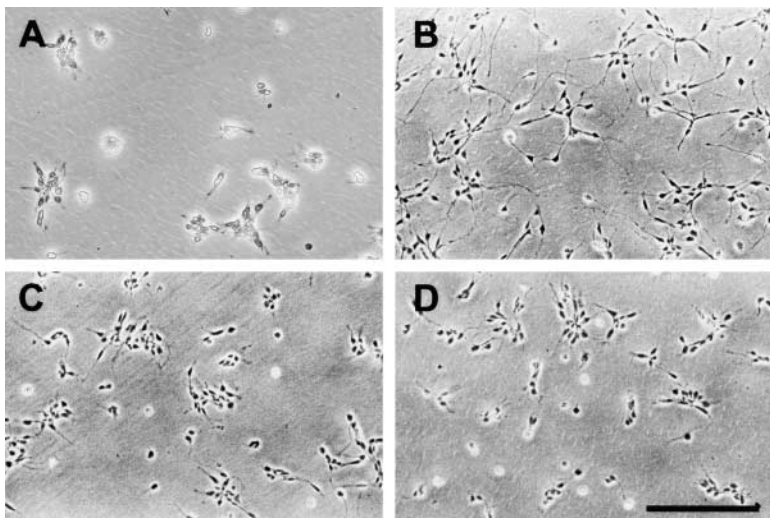
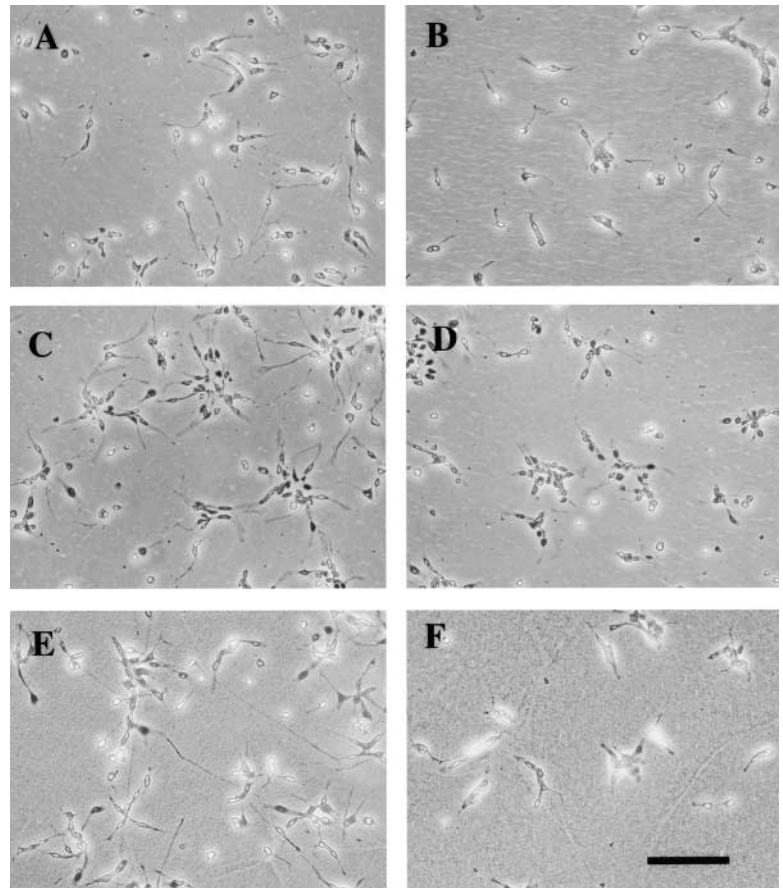


Figure 5. **cPTPRO inhibits LN-induced neurite outgrowth.** Dissociated E6 chick retinal neurons were allowed to grow overnight, fixed, and photographed through a phase-contrast microscope. (A) PDL alone, (B) PDL plus 10  $\mu$ g/ml LN, (C) PDL plus LN/50  $\mu$ g/ml cPTPRO-Fc, or (D) PDL plus LN/100  $\mu$ g/ml cPTPRO-Fc. Neurons grew long neurites on PDL-LN. Neurites were noticeably fewer and shorter when cPTPRO was mixed with LN, especially at the higher concentration (D). Bar, 100  $\mu$ m.

**Figure 6. cPTPRO inhibits neurite outgrowth on several growth-promoting substrates.** Neurite growth was pronounced on 2- $\mu$ l spots of 100  $\mu$ g/ml rat tail collagen I (A), 20  $\mu$ g/ml fibronectin (C), and 20  $\mu$ g/ml N-cadherin-Fc (E). In contrast, when 100  $\mu$ g/ml cPTPRO-Fc was mixed with collagen, fibronectin, or N-cadherin-Fc, neurite outgrowth was noticeably reduced (B, D, and F, respectively). Bar, 100  $\mu$ m.



The inhibitory effect of cPTPRO on retinal neurite growth could also be demonstrated for growth induced by proteins other than LN. Several other extracellular matrix proteins, including collagen I and fibronectin, also promote neurite growth from retinal neurons. We found that the cPTPRO ECD was inhibitory to neurite growth induced by either of these two proteins (Fig. 6, A–D). Further, the ability of cPTPRO to inhibit neurite growth was not limited to extracellular matrix inducers, as cPTPRO also strongly inhibited neurite growth induced in retinal neurons by N-cadherin (Fig. 6, E and F). Quantification of neurite growth data demonstrated that cPTPRO inhibition of neurite growth was statistically significant when either the percentage of neurons with neurites (Fig. 7, C–E) or the length of individual neurites (unpublished data) was considered. The ability of cPTPRO to inhibit neurite growth induced by this variety of substrate proteins suggests that the effects are not due to binding of cPTPRO to the inducing protein, or by binding of cPTPRO to the neuronal receptors for these inducers. Rather, the data suggest that cPTPRO delivers an inhibitory signal to the cell by interacting with its own receptor.

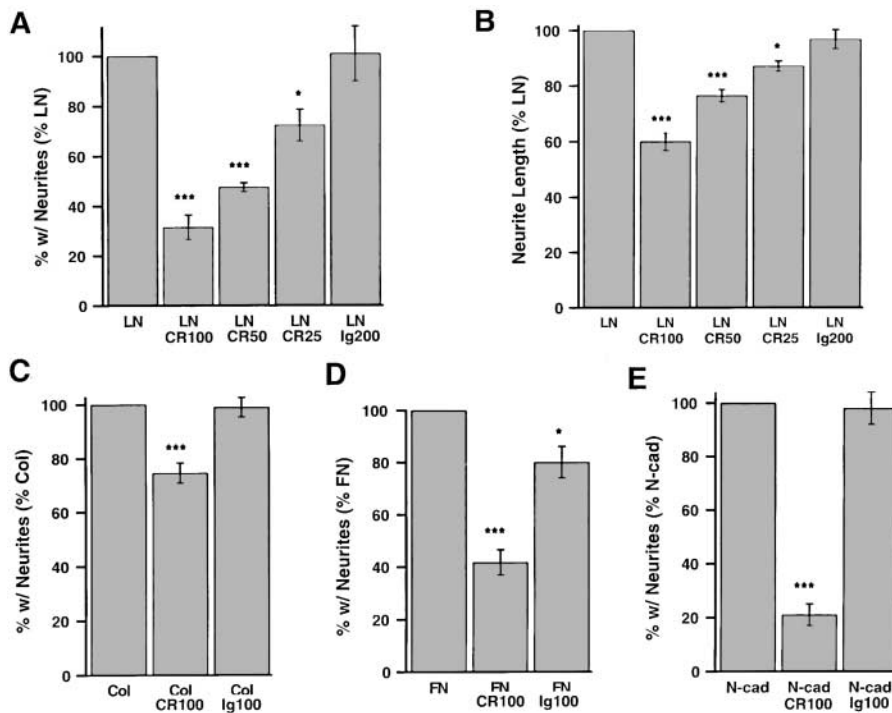
#### **cPTPRO induces retinal growth cone collapse**

Proteins that inhibit neurite outgrowth by interacting with specific inhibitory receptors are candidates for repulsive axonal guidance cues. Such repulsive guidance cues may be assayed *in vitro* using growth cone collapse assays. For example, several of the best-known families of repulsive guidance proteins, including semaphorins, ephrins, slits, and bone

morphogenetic proteins, exhibit growth cone collapsing activity *in vitro* (Luo et al., 1993; Drescher et al., 1995). Therefore, we tested the growth cone collapsing ability of cPTPRO to determine whether cPTPRO might also act as a repulsive guidance cue. In these assays, we added various concentrations of soluble cPTPRO-Fc (or mIgG-1 as a control) to cultures of E6 retinal explants 30 min before fixation. We found that concentrations of cPTPRO as low as 60 pM had clear collapsing ability, and that this activity was already saturated at 0.6 nM (Fig. 8). The potency of the cPTPRO ECD in collapsing growth cones, as well as the percentage of growth cones collapsed at saturating cPTPRO concentrations, were similar to the values seen previously for ephrin A5-Fc, another repulsive guidance cue for retinal neurons (Wahl et al., 2000), and for the collapse of dorsal root ganglia growth cones by semaphorin IIIA (Luo et al., 1993).

#### **cPTPRO is a repulsive guidance cue for retinal axons**

The ability of the cPTPRO ECD to inhibit neurite growth when present on the substrate, combined with its growth cone collapsing activity, suggests the possibility that cPTPRO is a repulsive guidance cue for retinal neurons. To test this possibility directly, we used a growth cone steering assay designed to examine growth cone guidance *in vitro*. This assay has been used to demonstrate growth cone steering by a variety of guidance proteins, including one type II RPTP (Gundersen and Barrett, 1980; Zheng et al., 1994; Ming et al., 1997; Song et al., 1997; Sun et al., 2000b). Be-



**Figure 7. cPTPRO inhibition of outgrowth induced by different substrates.** The percentage of neurons with neurites (A, C–E) or the mean length of neurites (B) was plotted for control substrates (LN; Col, Collagen I; FN, fibronectin; and N-cad, N-cadherin) and for substrates mixed with the indicated concentrations of cPTPRO–Fc (in  $\mu\text{g/ml}$ ). (A and B) cPTPRO–Fc inhibited neurite growth on LN in a dose-dependent manner. (A) The percentage of neurons with neurites was significantly reduced by cPTPRO at each concentration tested, but not by mIgG. (B) The length of retinal neurites was significantly reduced by cPTPRO. Inhibition of neurite outgrowth by cPTPRO–Fc was also apparent on a substrate of 100  $\mu\text{g/ml}$  collagen (B), 20  $\mu\text{g/ml}$  fibronectin (C), and 20  $\mu\text{g/ml}$  N-cadherin (D). A slight (19%) inhibitory effect was seen with high concentrations of IgG on fibronectin for unknown reasons. All plots show the mean results for four independent experiments  $\pm$  SEM as a percentage of the control condition

except D, which is the mean of three experiments.  $*P < 0.05$ ,  $***P < 0.001$ , significantly different from control. Statistics were done on InStat<sup>®</sup> using three-way analysis of variance and Bonferroni's posttest to compare each column to control.

cause both cPTPRO and its putative binding partner are expressed by RGCs, we wished to examine the influence of cPTPRO on the growth cones of RGC axons. Therefore, we used retinal explants rather than dissociated neurons for these experiments; the neurites leaving these explants are mainly the axons of RGCs (Barnstable and Drager, 1984; Akagawa and Barnstable, 1986; Sheppard et al., 1988; McLoon and Barnes, 1989). RGC growth cones were subjected to a stable gradient of soluble cPTPRO–Fc using the methods described previously for the type II RPTP, PTP- $\delta$  (Sun et al., 2000b). Within 15 min of the cPTPRO gradient being established, growth cones turned away from the source of the cPTPRO gradient (Fig. 9). Measurement of individual growth cone trajectories revealed that this repulsive turning response was quite consistent (Fig. 10 A), and quantification demonstrated an average turning angle of  $\sim -20^\circ$  ( $n = 22$ ; Fig. 10 B), similar to earlier data using semaphorin IIIA (Song et al., 1998). Control experiments with gradients of mIgG showed no turning effect, demonstrating that the repulsive effect of cPTPRO–Fc was due to the cPTPRO ECD (Fig. 10, A and B).

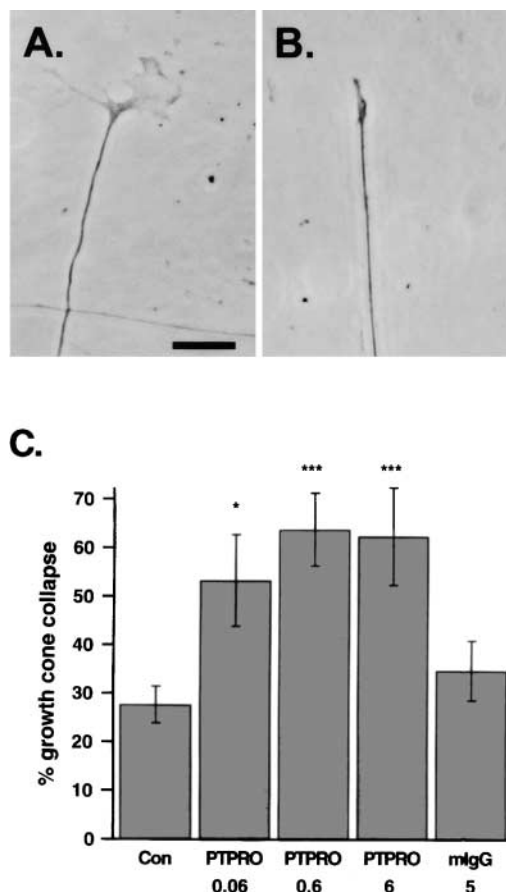
RGC neurites tend to extend in a clockwise direction on an LN substrate (Halfter et al., 1987), and this tendency could be confirmed in our experiments (unpublished data). To ensure that a clockwise bias could not explain the repulsive turning effects of cPTPRO–Fc, we performed assays in which the pipette tip was oriented either to the right (clockwise) or to the left (counterclockwise) of the migrating growth cone. In either case, a clear and significant repulsive tendency was observed ( $n = 10$  for each direction; unpublished data). Therefore, the cPTPRO ECD is a repulsive growth cone steering protein for RGC axons.

Poo and collaborators have shown that most growth cone steering molecules can be classified into two groups, depending on the sensitivity of the steering response to removal of extracellular  $\text{Ca}^{2+}$  and to changes in cyclic nucleotide levels (Ming et al., 1997; Song et al., 1997, 1998). For group I proteins (e.g., netrin, brain-derived neurotrophic factor [BDNF], myelin-associated glycoprotein [MAG]), attractive steering responses can be converted to repulsion by reducing intracellular cAMP activity, and repulsion can be converted to attraction by increasing cAMP. In group II (semaphorin III, NT-3) attractive responses can be converted to repulsion by reducing intracellular cGMP, and repulsion can be converted to attraction by increasing cGMP levels. We recently identified the ECD of a type II RPTP, PTP- $\delta$ , as an attractive growth cone steering protein; it belongs neither to group I nor to group II (Sun et al., 2000b). To determine whether the cPTPRO ECD belongs to group I or II, we increased cyclic nucleotide levels in retinal neurons before exposing them to gradients of cPTPRO–Fc. Application of Sp-cAMPs (to increase cAMP) had no effect on the repulsive activity of cPTPRO–Fc ( $n = 12$ ; Fig. 10, A and B). However, application of 8-Br-cGMP (to increase cGMP) converted cPTPRO-mediated repulsion into attraction ( $n = 13$ ; Fig. 10, A and B). Thus, the cPTPRO ECD can be classified as a repulsive group II guidance protein in vitro.

## Discussion

Our major finding is that the ECD of cPTPRO, a type III RPTP, is an antiadhesive and neurite inhibitory ligand for retinal neurons in vitro. Further, direct tests demonstrate that the cPTPRO ECD is a potent growth cone collapsing signal, and acts as a type II repulsive guidance cue for RGC





**Figure 8. cPTPRO-Fc induces collapse of retinal growth cones.** E6 chick retinal neurons were plated on glass coverslips coated with PDL and 10  $\mu\text{g/ml}$  LN. After 10 h of culture, cPTPRO-Fc or mlgG-1 was added to the wells in 100  $\mu\text{l}$  of medium. The same volume of medium was added to control wells. After 30 min, the cells were fixed and growth cones were scored as open or collapsed. (A) Control neurites had well-spread growth cones, in contrast to neurites treated with 6 nM cPTPRO-Fc (B). (C) Quantification of the growth cone collapsing effect. The percentage of collapsed growth cones (mean  $\pm$  SEM) was plotted for control neurons and neurons treated with various concentrations of cPTPRO-Fc or mlgG. Concentrations are given in nM. Neurons treated with cPTPRO-Fc showed significantly higher levels of growth cone collapse than controls. Bar, 10  $\mu\text{m}$ .

growth cones. Our findings comprise the first evidence for a functional effect of a type III RPTP on vertebrate neurons, and suggest that this class of RPTP operates in a manner distinct from the more widely studied type II RPTPs. Together with our data on the expression of cPTPRO, these results suggest that PTPRO has a functional role in vertebrate neural development.

A variety of independent assays confirm the inhibitory and/or repulsive effects of cPTPRO. The PTPRO ECD is antiadhesive and inhibits neurite growth when presented as a substrate protein. Similarly, soluble PTPRO collapses growth cones and is a specific repulsive steering signal when presented in a gradient. Furthermore, PTPRO inhibits growth induced by numerous distinct growth-promoting proteins, which in turn act through a diverse array of neuronal receptors. Therefore, it is unlikely that PTPRO exerts its inhibitory effect by direct interference with the binding functions of positively acting receptors. Rather, our results

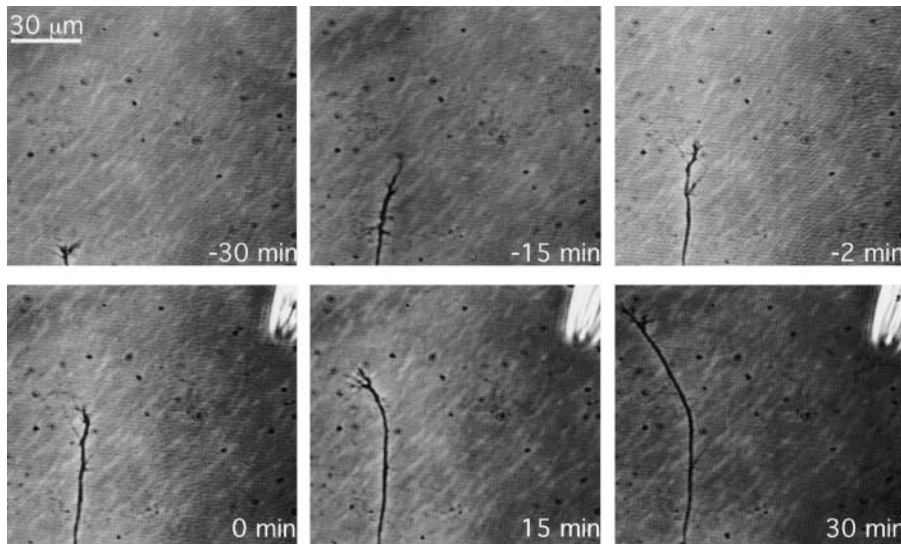
suggest that PTPRO acts through one or more specific receptors on the neuronal surface that send an inhibitory signal to the growth cone machinery.

PTPRO mRNA is selectively expressed in the brain and kidney, and is expressed most strongly during embryonic development (Thomas et al., 1994; Bodden and Bixby, 1996; Tagawa et al., 1997; Chilton and Stoker, 2000). Our protein expression data are in agreement with these observations and indicate that cPTPRO is mainly expressed in the forebrain from E7 to E14, the major period of axonogenesis in the chick (Rogers, 1957; De Long and Coulombre, 1965). Similarly, cPTPRO in the retinotectal system is highly expressed during development, and concentrated on the axons of projection neurons, both in the retina and in the tectum (Ledig et al., 1999b). PTPRO is also likely to be important in development in other areas of the nervous system. Recent expression studies have found PTPRO mRNA in several types of brain projection neurons and most peripheral ganglia during mouse development (unpublished data).

The ectodomains of numerous type II RPTPs, as well as the type V RPTP PTP- $\zeta$ , have been shown to be adhesive, neurite growth-promoting molecules in vitro (Maeda and Noda, 1996; Burden-Gulley and Brady-Kalnay, 1999; Drosopoulos et al., 1999; Garwood et al., 1999; Ledig et al., 1999a; Revest et al., 1999; Wang and Bixby, 1999). In some circumstances the PTP- $\zeta$  ECD, which is a chondroitin sulfate proteoglycan, can also act as a neurite inhibitory ligand (Maeda and Noda, 1996). Our results demonstrate that the ECD from a type III RPTP can provide a neurite inhibitory and growth cone repellent signal to neurons. The only other RPTP that has been shown to steer growth cones is PTP- $\delta$ , which is a growth cone attractant whose activity is not influenced by changes in cyclic nucleotide levels. The intracellular signaling pathway triggered by PTPRO remains a mystery. Growth cone steering experiments indicate that cGMP plays a modulatory role, but there is no evidence that this is a primary element of the signaling pathway. In the case of group I (cAMP-regulated) guidance cues, it appears that distinct cues operate through shared signaling pathways, including the phosphatidylinositol 3-kinase and PLC- $\gamma$  pathways (Song and Poo, 1999). If this is also true for group II (cGMP-regulated) guidance cues, observations on the other group II proteins, semaphorin 3A and NT-3, might be relevant. The signaling pathway of semaphorin 3A, which is the only other group II chemorepellent, appears to involve both Rac1 and collapsin response mediator proteins (Nakamura et al., 2000). NT-3 signaling has been linked to the Ras-mitogen-activated protein kinase pathway and the PLC- $\gamma$  pathway in hippocampal pyramidal neurons (Marsh and Palfrey, 1996).

There are no known ligands for cPTPRO or any type III RPTP. Several type II RPTPs bind homophilically, including PTP- $\mu$ , PTP- $\kappa$ , PTP- $\delta$ , and HmLAR2 (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994; Wang and Bixby, 1999; Baker et al., 2000). Other type II RPTPs are presumed to have heterophilic ligands and/or receptors, including CRYP- $\alpha$ /PTP- $\sigma$  (Haj et al., 1999; Ledig et al., 1999a). Our data for PTPRO also suggest a heterophilic ligand(s). First, cPTPRO and its presumptive binding partner exhibit differential staining patterns in the developing





**Figure 9. A growth cone from an embryonic chick RGC turns away from a source of cPTPRO-Fc during the application of a cPTPRO-Fc gradient.** The growth cone was migrating in a vertical direction before gradient onset, but turned away from the pipette (upper right corner) within 15 min of gradient onset, and continued to move away from the source over the next 15 min. Times are given relative to onset of the gradient.

retina. Second, the cPTPRO ECD is not adhesive for neurons expressing cPTPRO. The nature of ligand–receptor interactions for cPTPRO is still unclear. PTPRO binding partners may act as receptors for the PTPRO ECD, as ligands for the PTPRO receptor, or both. Because there is overlap in the expression patterns of cPTPRO and its putative ligand, we must also consider the possibility that these interactions occur in a “cis” fashion within the plane of the plasma membrane. A transmembrane protein known as gp150 has been characterized as a substrate and potential cis binding partner for PTP10D, which is the closest *Drosophila* homologue of PTPRO (Fashena and Zinn, 1997). The functional significance of gp150–PTP10D interactions is not yet known.

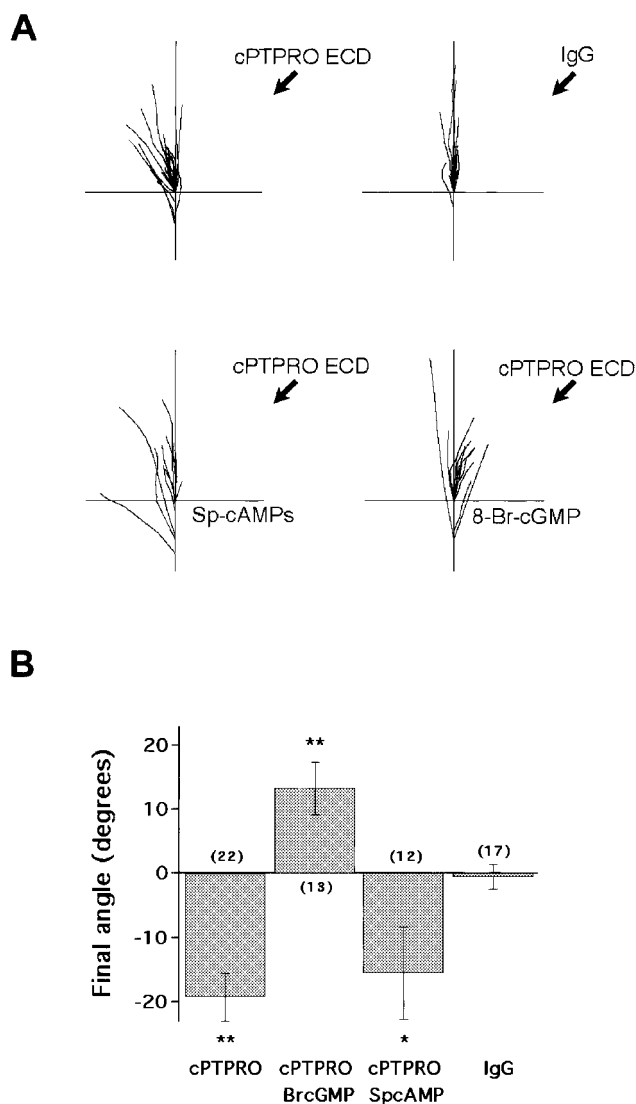
Genetic knockout experiments in *Drosophila* suggest that RPTPs play key roles in axon growth and guidance, but also suggest complex interactions among these proteins as well as some functional redundancy. For instance, flies with a mutation in PTP10D have no obvious nervous system abnormalities. Similarly, flies lacking PTP69D have no clear central nervous system phenotype. However, mutants that lack both PTP10D and PTP69D exhibit abnormal midline crossing by a subset of longitudinal axons. When all four nervous system–specific RPTPs are knocked out, this phenotype is greatly exacerbated (Sun et al., 2000a). In contrast, whereas removing the type II RPTP Dlar causes some motor axons to bypass their target, removal of a second RPTP, DPTP99A, tends to rescue the phenotype. A similar complexity is likely to exist in the vertebrate. Genetic deletions of RPTPs in mice have yielded animals with central nervous system abnormalities (Yeo et al., 1997; Elchebly et al., 1999; Wallace et al., 1999; Harroch et al., 2000; Uetani et al., 2000), but the roles of these proteins in axon growth will likely be clear only through more intensive analysis and examination of double and triple knockouts. The function of PTPRO in axon growth in vivo is not yet known. Other proteins shown to act as repellent growth cone steering proteins in vitro have generally been found to guide axons in vivo. A detailed examination of the nervous system in the PTPRO knockout mouse (Wharram et al., 2000) should provide clear information on this issue.

It is interesting that the ECDs of PTP- $\delta$  and PTPRO were found to have such different effects on neuronal adhesion and axon growth. The PTP- $\delta$  ECD mediates neuronal adhesion, stimulates neurite growth, and is an attractive guidance cue in vitro (Wang and Bixby, 1999; Sun et al., 2000a). The PTPRO ECD is antiadhesive, inhibits neurite growth, and is a group II repulsive guidance cue in vitro. PTP- $\delta$  and other type II RPTPs have the extracellular structures of Ig superfamily CAMs, thus their positive effects on axon growth might be predicted. On the other hand, Eph receptors have ECDs consisting of one Ig domain and two fibronectin type III repeats (Pandey et al., 1995), and many Eph receptor–ephrin interactions are neurite inhibitory (Drescher et al., 1997; Mellitzer et al., 2000). No such precedents exist for type III RPTPs, as ECDs comprising only multiple fibronectin type III repeats are not known for other protein families. However, the functional differences seen in our studies may relate to a genetic antagonism seen among RPTPs in *Drosophila*. The motor axons in the intersegmental nerve terminate without reaching normal branch points in embryos lacking three RPTPs, including two type IIa RPTPs (Dlar and PTP69D). If PTP10D (a cPTPRO homologue) is removed from flies in this background, the phenotype is partially rescued, suggesting that PTP10D acts in opposition to the other RPTPs in this context (Sun et al., 2001). Our finding that cPTPRO, unlike vertebrate type II RPTPs, acts as a neurite inhibitory ligand, suggests a potential functional explanation for this genetic antagonism.

## Materials and methods

### Materials

Fertilized White Leghorn eggs were purchased from Spafas and incubated at 38°C in a humidified chamber until use. Cell culture media were purchased from University of Miami Sylvester Comprehensive Cancer Center Cell Culture and Media Supplies Facility. CHO-K1 cells were a gift from Dr. Kerry Burnstein (University of Miami, Miami, FL). An N-cadherin–Fc fusion protein was produced and purified by cloning the entire ECD of chick N-cadherin into the Fc fusion vector already described, and using the purification methods described (Wang and Bixby, 1999). Recombinant N-cadherin–Fc had neurite growth–promoting ability similar to that seen with purified native N-cadherin (unpublished data). All other reagents were purchased from Sigma-Aldrich unless specified.



**Figure 10. Summary of growth cone turning in response to cPTPRO.** (A) Superimposed traces of growth cone trajectories in response to gradients of either cPTPRO-Fc or mlgG-1. The origin represents the position of the growth cone at the gradient onset, and the vertical line represents the original direction of growth (before gradient onset). Large arrows represent the position of the pipette at  $\sim 45^\circ$  to the original direction of growth. Almost all growth cones tested turned away from the source of cPTPRO-Fc. In contrast, growth cones maintained their original heading in the presence of an IgG gradient. Addition of Sp-cAMPs had no effect on the repulsive effect of cPTPRO, but the addition of 8-Br-cGMP converted cPTPRO-mediated repulsion into attraction. (B) Quantification of the mean final turning angle ( $\pm$  SEM) of the growth cones after a 1-h gradient application. The repulsive effects of cPTPRO-Fc, and the attraction in the presence of 8-Br-cGMP, are clearly shown. \* $P < 0.05$ , significantly different from IgG control; \*\* $P < 0.01$ , significantly different from control. Numbers in parentheses indicate the number of growth cones examined.

### Expression and purification of cPTPRO-Fc

A unique SpeI site was introduced at the 3' end of a cDNA fragment encoding the entire ECD of cPTPRO (Bodden and Bixby, 1996) by PCR. This product was cloned in frame, upstream of the Fc region of mouse IgG-1, and subcloned into the pcDNA3 expression vector as described (Wang and Bixby, 1999). CHO-K1 cells were stably transfected with purified

cPTPRO-Fc as described (Wang and Bixby, 1999). Established cell lines were screened for expression by Western blot analysis of conditioned media with anti-cPTPRO antibody. Purification was performed as described, except that 0.5  $\mu$ M of the protease inhibitor PMSF was added into the conditioned medium before passing it through the anti-mlgG-1 column. Western blotting for cPTPRO and the fusion protein was performed essentially as described previously (Bixby and Jhabvala, 1990). The primary antibody comprised 0.5  $\mu$ g/ml of anti-CRYP-2 IgG, immunopurified from an antiserum raised against a CRYP-2-GST fusion protein that has previously been characterized (Ledig et al., 1999b).

### Immunohistochemistry

Frozen 10- $\mu$ m sections of E7 chick retina were prepared as described (Ledig et al., 1999b). For binding experiments, 5  $\mu$ g/ml cPTPRO-Fc or 10  $\mu$ g/ml mlgG-1 was allowed to bind to the sections overnight. The sections were rinsed and 1:250 dilution of goat anti-mouse IgG was added, followed by 1:250 dilution of anti-goat-HRP. Sections stained for cPTPRO were treated with the rabbit anti-CRYP-2 antibody followed by anti-rabbit-HRP. HRP immunohistochemistry was performed with diaminobenzidine-nickel substrate.

### Adhesion assays

Cultures of dissociated neurons from E6 retina were prepared as described (Perron and Bixby, 1999). Retinal cultures are  $>70\%$  neuronal by the criterion of neurite outgrowth and neurofilament expression. Neuronal adhesion assays were performed as described (Wang and Bixby, 1999). For sequential spotting experiments, 1  $\mu$ l of cPTPRO (50  $\mu$ g/ml) or mlgG was spotted onto the dish. After incubation for 15 min, the remaining protein was removed by pipette and 10  $\mu$ l of LN (10  $\mu$ g/ml) was spotted so as to cover and surround the first spot. Neurons were plated at  $5 \times 10^5$  cells/35-mm dish and allowed to adhere for 2 or 24 h before fixation. For simultaneous spotting experiments, 20  $\mu$ g/ml LN or 10  $\mu$ g/ml N-cadherin-Fc was mixed on ice with cPTPRO-Fc or control mlgG immediately before spotting on the dish.

### Neurite outgrowth assays

Assays were performed as described previously (Bixby and Jhabvala, 1990). Proteins were spotted on an LN- or PDL-coated dish, except for N-cadherin experiments which used a nitrocellulose substrate. After 1 h, dishes were rinsed and blocked with culture medium. Neurons were allowed to grow 14–16 h before fixation. Using NIH Image software, video images of the cultures were used to measure average neurite lengths only in cells in which neurites were longer than twice the cell body diameter. At least 100 neurites were measured per spot of protein. For the percentage of neurons with neurites, all cells within the protein spot (at least 500 cells/spot) were counted.

### Growth cone collapse assay

Collapse assays followed the protocol of Goshima et al. (1995). Retinal explants were plated on LN-coated glass coverslips in 24-well plates in 250  $\mu$ l of culture medium. After 10 h, cPTPRO-Fc or mouse IgG-1 as a control was added in a small amount of retinal culture medium, and the cells were incubated at 37°C for 30 min before fixation. Growth cones were scored as collapsed or not collapsed, and those with an intermediate morphology were not counted.

### Growth cone steering assay

The growth cone steering assay was performed on neurites emanating from explants of E6 retina essentially as described for forebrain neurons (Sun et al., 2000b). Explants were plated on a collagen substrate 1 d before the experiment. CPTPRO-Fc was added to the micropipette at a concentration of 10  $\mu$ g/ml. The initial concentration experienced by the growth cone at the onset of the gradient was calculated to be  $\sim 400$  ng/ml (1 nM; Sun et al., 2000b). The final turning angle was defined as the angle between the original direction of neurite extension and a straight line connecting the position of the growth cone at the beginning and end of the experiment. For experiments with cyclic nucleotides, 20  $\mu$ M Sp-cAMP or 100  $\mu$ M Br-cGMP was added at least 30 min before the recording period.

### Digital images

Digital images were prepared with Adobe Photoshop® (v5.02).

### Statistics

Statistics were done using InStat® (v2.03).

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