The Receptor Tyrosine Phosphatase C R Y P α Promotes Intraretinal Axon Growth

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Abstract. Retinal ganglion cell axons grow towards the optic fissure in close contact with the basal membrane, an excellent growth substratum. One of the ligands of receptor tyrosine phosphatase C R Y P α is located on the retinal and tectal basal membranes. To analyze the role of this RPTP and its ligand in intraretinal growth and guidance of ganglion cell axons, we disrupted ligand-receptor interactions on the retinal basal membrane in culture. Antibodies against C R Y P α strongly reduced retinal axon growth on the basal membrane, and induced a dramatic change in morphology of retinal growth cones, reducing the size of growth cone lamellipodia. A similar effect was observed by blocking the ligand with a C R Y P α ectodomain fusion protein. These effects did not occur, or were much reduced, when axons were grown either on laminin-1, on matrigel or on basal membranes with glial endfeet removed. This indicates that a ligand for C R Y P α is located on glial endfeet. These results show for the first time in vertebrates that the interaction of a receptor tyrosine phosphatase with its ligand is crucial not only for promotion of retinal axon growth but also for maintenance of retinal growth cone lamellipodia on basal membranes.

Key words: receptor protein tyrosine phosphatase • axon growth • actin cytoskeleton • growth cone • integrin

One of the first steps in retinal axon growth occurs early in development in the retina. During the process of intraretinal axon guidance, ganglion cell axons grow towards the optic fissure and exit the eye at the optic nerve head to reach their visual centers in the brain. In the chick embryo, the first retinal ganglion cell axons are found in the central retina at Hamburger-Hamilton (HH) stage 15 (E2-E3), shortly after invagination of the optic vesicle (Mey and Thanos, 1992). From the earliest stages onwards these axons grow straight to the optic fissure. A more and more ganglion cells differentiate and send out axons, fascicles form which also head directly to the fissure. In recent years, several mechanisms have been suggested to account for intraretinal axon guidance.

Neurelin, the goldfish homologue of D M - G R A S P , is involved in guidance of dorsal retinal axons towards the optic disc (Ott et al., 1998). Neurelin is a member of the immunoglobulin superfamily consisting of five extracellular Ig domains, a transmembrane region, and a short cytoplasmic sequence. In vivo injections of monoclonal antibodies against Ig domains 1 and 3 resulted in defasciculation of retinal axons, whereas antibodies directed against Ig domain 2 led to intraretinal guidance errors of ganglion cell axons (Leppert et al., 1999). The direct growth towards the optic disc also requires other immunoglobulin superfamily members like L1 and N-CAM (Brittis and Silver, 1995; Brittis et al., 1995). Injection of Fab fragments against E587, a member of the L1 subfamily, resulted in a delay of fasciculation of young axons as well as defasciculation (Bastmeyer et al., 1995; Giordano et al., 1997).

A role for the FGFR receptor (FGFR), a receptor tyrosine kinase, in guidance of retinal ganglion cell axons towards the fissure became evident after adding receptor-specific antibodies to living whole-mount rat retinae. Blocking of the FGFR resulted in defasciculation of retinal axons in the center of the retina and in guidance errors in the retinal periphery (Brittis et al., 1996). In Xenopus retinae, in >40% of retinal ganglion cells expressing a truncated, kinase-defective FGFR, axons were unable to leave the retina (McFarlane et al., 1996). These observations underscore the importance of the FGFR in intrareti-
nal axon guidance and in the exit of retinal axons from the eye. The results are at least partially explained by the function of this receptor in mediating the effects of N-cadherin, NCA M, and L1 (reviewed in Viollet and Doherty, 1997), other cell adhesion molecules and components of the ECM.

Chondroitin sulfate proteoglycan, a major component of the ECM, was suggested to act as an inhibitory molecule, preventing growth of retinal axons towards the periphery (Brittis et al., 1992). Having reached the optic disc, axons start to exit from the eye to enter the optic nerve. In the mouse embryo, netrin-1 seems to be responsible for the exit of retinal axons but not for their growth towards the optic disc (Deiner et al., 1997). In the eye, retinal axons grow in close contact to an extracellular matrix structure, the basal lamina or basal membrane (BM) (Rager, 1980; Eas-ter et al., 1984; Silver and Rutishauser, 1984; Halfter and Boxb erg, 1992; H alfter 1996). The retinal basal lamina (membrana limitans interna) is an excellent growth substrate for retinal axons and is superior to laminin (H alfter, 1996) and, moreover, extremely important during early stages of neural development (H alfter, 1998). Its outgrowth promoting activities, however, are not completely characterized. Important molecular components of this structure include laminin, nidogen, collagen IV, agrin, heparan sulfate proteoglycan, chondroitin sulfate proteoglycan, tenascin, and at least 10 unidentified extracellular matrix components (Halfter and Boxb erg, 1992; H alfter, 1996; Faissner, 1997). Among these unidentified molecules are probably candidates contributing to the excellent growth-stimulating characteristics of the retinal BM.

We became interested in studying the retinal basal membrane in more detail because of the recent finding that several receptor protein tyrosine phosphatases (RPTPs)1, including CRYP a (Stoker, 1994), CRYP b (Boden and Bixby, 1996), and PTP e (Gebbink et al., 1991), are expressed on retinal axons and growth cones during formation of the retinotectal projection (Stoker et al., 1995a; Ledig et al., 1999). CRYP a is a member of the type IIa subfamily of RPTPs. This subfamily is of particular interest given their structural resemblance to the neural CAMs (see Fig. 1) and their expression pattern in the developing CNS (Schaaveld et al., 1998). This structure suggested that they would have extracellular ligands either on cells or in the extracellular matrix. A though the identity of these ligands remains to be determined, it appears that at least one of these is present on the retinal BM (Haj et al., 1999).

To date the function of these RPTPs during nervous system development in vertebrates remains largely to be determined (Stoker and Dutta, 1998; van Vactor, 1998). Most of what we know about the function and signaling mechanisms of RPTPs comes from studies in Drosophila (Desai et al., 1997a). The Drosophila RPTPs, D L a R, D P T P 69 d, and D P T P 9 9 a are required for motor axon guidance (Desai et al., 1996, 1997b; Krueger et al., 1996). Signals downstream of DLAR also control the actin cytoskeleton of axons (Willis et al., 1999a,b). In vertebrates, very recent data (U etani et al., 1997; Eichbly et al., 1999; Wallace et al., 1999) also indicate that at least PTP e (CRYP a is the chick orthologue of PTP e) and PTP e (Mizu no et al., 1993) are important for proper development of the central nervous system. PTP e, belonging to the same type II family of PTPs as CRYP a, was also recently described as being important for regulating cadherin-mediated retinal axon outgrowth (Burden-Gulley and Brady-Kalnay, 1999).

We have addressed the question of CRYP a function in early retinal axons by attempting to perturb functionally the interactions of CRYP a with its ligand in the in vivo substrate, the retinal BM. The presence of a BM ligand was demonstrated in vitro using a fusion protein consisting of the ectodomain of CRYP a and the enzyme alkaline phosphatase (α1-AP) (see Fig. 1 for structures). This protein prominently binds to the BM (Haj et al., 1999). To analyze the potential function of the CRYP a ligand, we grew retinal axons on retinal BMs (H alfter et al., 1987) and treated them either with CRYP a specific antibodies or with soluble α1-AP. Both the axonal growth rate and the growth cone morphology of retinal neurons were strongly influenced by these reagents. Significantly, on BMs with endfeet removed (BM-Ef) and on an artificial acellular BM (matrigel), these effects were sharply reduced. We conclude that CRYP a and its ligand on glial endfeet play important roles in promoting retinal axon growth and maintenance of growth cone morphology.

**Materials and Methods**

**Antibodies and Reagents**

The generation of the polyclonal antibody anti-CRYP a and its purification have been previously described (Stoker et al., 1995a). The polyclonal anti-NCA M antibody and its function-blocking activity have been characterized (Bixby and Richardt, 1987; Bixby et al., 1987). The monoclonal antibody against β1-integrin was JG22 (Greve and Gottlieb, 1982; Tomasetti et al., 1986). The CRYP a 1-AP and 1-316 fusion proteins were generated by subcloning CRYP a 1 fragments spanning amino acids 1-721 and 1-316 into the APtag2 vector (Cheng et al., 1995; Haj et al., 1999). AP was generated by transfecting the A PTaq4 vector (Cheng et al., 1995). All three vectors were transfected into cos7 or 293T cells using Superfect (Qiagen) and the proteins were collected after 6 d in the conditioned medium. Medium was passed through an anti-AP agarose column (Sigma), and the fusion protein eluted using 0.1 M glycine-HCl, pH 2.5, with immediate buffering to pH 8 with Tris-HCl. The protein was dialyzed against TBS buffer and stored at 4°C. Laminin-1 and matrigel were obtained from Becton Dickinson. N-cadherin was purified as described (Bixby and Zhang, 1990).

**AP Staining**

E6 retinal cryosections were prepared as previously described (Ledig et al., 1999) and incubated with α1-AP. The AP staining method was also previously described (Cheng et al., 1995; Haj et al., 1999).

**Culture of Retinal Explants: The Basal Membrane Assay**

The basal membrane assay was performed as described in H alfter et al. (1987). The basal lamina was prepared from E7 retina. In the first step, the retina is flattened on a nitrocellulose filter (Sartorius AG). The filter with the attached retina is then put upside down on a poly-L-lysine (PLL)-coated glass coverslip (see Fig. 2 A). Two small metal bars are put...
on the filter before it is incubated for 10 min at 37°C. Aferwards the filter is lifted up while the basal lamina sticks to the glass surface (see Fig. 2 B). To remove the glial endfeet from the basal lamina, glass coverslips were washed with 2% Triton X-100 for 5 min. The Triton was removed after three 10-min wash steps with Hank’s solution. For experiments with laminin as a growth substrate glass coverslips were coated with laminin at 37°C for 2 h and afterwards washed with Hank’s. Coating of glass coverslips with matrigel was carried out according to the manufacturer’s protocol. For experiments with N-cadherin as a growth substrate, PLL-precoated glass coverslips were coated with N-cadherin at 37°C for 3 h, resulting in a PLL-N-cadherin substrate mix. Retinal explants as outgrowth source were prepared from E6 retinas as previously described (Ledig et al., 1999). Cultures were incubated for 24 h at 37°C in 1 ml F12 medium containing methylcellulose and various amounts of preadded different antibody in a humidified chamber (5% CO2).

Staining of Retinal Cultures

Afer incubation cultures were immediately fixed in 4% paraformaldehyde (dissolved in 0.1 M phosphate buffer) for 10 min at room temperature. They were permeabilized with 0.1% Triton X-100 in PBS and blocked by 1% BSA in PBS. Stainings with Alexa-labeled phalloidin were performed according to the manufacturer’s protocol (Molecular Probes Inc.). The fixed cultures were finally covered with moviol.

Evaluation of Data

A nalysis was done using an Axiopt (Zeiss) fluorescence microscope using a Sony CCD-camera together with the Analysis program (SiS). To quantify outgrowth real-time pictures were taken and directly measured on the screen. The average axon length of a culture was determined as the distance from the explant to the region reached by at least 60% of the axons (longer and shorter neurites were not considered) (see Fig. 3 A). Growth cone morphology was analyzed by measuring two diameters of a growth cone using a 100× objective (see Fig. 3 B). The d1 parameter represents the length of the growth cone in μm, measured from the growth cone-axon neck to the border of the leading lamellae, and the d2 parameter represents its width, characterized mainly by the extension of its lamellipodia. The data were analyzed by regression analysis, ANOVA, and Student’s t test using the EXCEL 98 program (Microsoft) and StatView 4.5 (Abacus Concepts Inc.).

Results

The CRYPα Ligand Is Expressed in the Retinal Basal Membrane

Immunostaining with CRYPα-specific antibodies strongly labels retinal axons inside the retina, optic nerve, and tract, and in the optic tectum of the chick embryo (Stoker et al., 1995b; Ledig et al., 1999). CRYPα is already present at E4 on retinal axons and growth cones. Two isoforms of CRYPα, CRYPα1 and α2 (Fig. 1), were described, both containing 3 Ig-like domains but differing in the number of fibronectin type III-like domains (FNIII). α1 has four and α2 has eight FNIII repeats, respectively (Stoker et al., 1995b). Until E7, only CRYPα1 is expressed in retinal ganglion cells, whereas CRYPα2 is coexpressed by E8 (Haj et al., 1999). We suggest, therefore, that for the early stages of retinal optic fiber growth, CRYPα1 is the relevent isoform. To examine the expression of potential ligands of this PTP, the extracellular domain of the CRYPα1 isoform was fused to the enzyme alkaline phosphatase (Fig. 1) and the fusion protein α1-A P was used to analyze the localization of the ligands in the developing chick retinotectal system (Haj et al., 1999). Using α1-A P (see Fig. 4 A), a ligand was detected at E6 in the basal membrane of the retina (see Fig. 4 B), the optic stalk and the optic chiasm (Haj et al., 1999) and in many basal membranes throughout the embryonic brain (Stoker, A.W., unpublished observations).

Blocking CRYPα-Ligand Interactions Reduces Retinal Axon Length

The expression of the putative ligand in the retinal basal membrane prompted us to use this membrane as a growth substrate for retinal axons and to analyze what role the interaction of CRYPα with its ligand has in retinal axon growth. To this end, retinal basal membranes were iso-
of portantly, the IG2 antibody is known to block the binding AP, thereby disturbing ligand-receptor interactions. Im-
cone. Bars: (A) 0.1 mm; (B) 0.01 mm.

Table 64x404 to 280x750

Figure 3. Criteria for data analysis. A shows a control culture on BM after a 24-h incubation time. We defined the average axon outgrowth as the distance between the explant and the growth front covered by at least 60% of the axons. B demonstrates how growth cone data were measured. A growth cone grown on LN was characterized by its two diameters d1 and d2. The arrow indicates lamellipodia and the arrowhead filopodia of the growth cone. Bars: (A) 0.1 mm; (B) 0.01 mm.

lated according to a published method (Halfter et al., 1987) and retinal strips were explanted. Retinal axons were grown on retinal basal membranes or on laminin for 24 h either in the presence of polyclonal antibodies directed against the two outermost Ig-like domains of CRYP (IG2 antibody; Fig. 1), or in the presence of α1-AP, thereby disturbing ligand-receptor interactions. Importantly, the IG2 antibody is known to block the binding of α1-AP to the basal membrane ligand (see Haj et al., 1999) and therefore is predicted to block CRYP ligand interactions in general. A fusion protein containing the 3 Ig domains of CRYP (Fig. 1; Ig3-AP) was used as a negative control, as previous experiments suggested that this protein does not to bind to the CRYP ligand (Haj et al., 1999). To analyze the effect of disturbing the CRYP ligand interaction, the average length of retinal axons leaving the explant was measured from the edge of the explant to the front of the majority of axons (Fig. 3 A).

The IG2 antibody and α1-AP strongly reduced the

length of retinal axons, in a dose-dependent manner on BM (Fig. 5, A and C). We obtained the same result in preliminary studies with IG2 antibodies to the tectal membrane stripe assay (Walter et al., 1987) with tectal membrane preparations (Ledig, M.M., and B.K. Mueller, unpublished data). Outgrowth of both nasal and temporal axons was reduced, but there was no effect on the decision behavior (data not shown). 125 pmol of IG2 reduced retinal axon length by 63% on basal membrane and by 25% on LN (Fig. 5, A and B). 26 pmol α1-AP reduced retinal axon length on BM by ~50% (Fig. 5 C). No comparable effect was observed with Ig3-AP on BM (data not shown) or with α1-AP or Ig3-AP when retinal axons were grown on LN (Fig. 5 D). A slight dose-dependent effect, caused by the alkaline phosphatase (AP), was observed on both LN and retinal BM, but only when used at high levels (compare Fig. 5, C and D, with controls in A and B). 125 pmol of NCA M antibody was used as a control for antibody binding and did not influence retinal axon length on LN or BM (Figs. 5, E and F, respectively). Moreover, there were also no obvious effects on fasciculation by IG2 or by the polyclonal control antibody NCA M (data not shown).

A second antibody, directed against the β1-integrin chain (JG22), was used to compare the effect of CRYP1 to one of the major receptors for ECM molecules. Using this antibody significantly reduced retinal axon length in a dose-dependent manner on both substrates. 125 pmol of JG22 blocked retinal axon growth on LN almost completely (Fig. 5 F). On BM retinal axon length was reduced by 71% (Fig. 5 E). This suggests the presence of different outgrowth promoting activities in basal membranes, mediating their effects by at least two different receptor sys-


tems, the integrins, and the RPTP CRYPα.

Blocking CRYPα Ligand Interactions Alters Retinal Growth Cone Morphology

The d1 and d2 parameters were used to measure growth cone extensions (Fig. 3 B). Retinal axons growing on the retinal basal membrane or on laminin exhibit a morphology with few filopodia but elaborate lamellipodia (Fig. 6 A). In the presence of increasing amounts of IG2 or α1-AP, lamellipodia are gradually retracted and the number of long filopodia is increased (Fig. 6, B–D and F–H). This striking transition from a lamellipodial to a filopodial growth cone morphology was most evident after adding 125 pmol IG2 or 26 pmol of α1-AP (Fig. 6, D and H) (Table I). Significantly, this was only observed on retinal BM (Figs. 6, A–H, and 9 A) but not on LN (Figs. 6, I–P, and 9 B). The transition in growth cone morphology was re-


dected in a ~40% reduction of the growth cone width (d2)

without any significant change of its length (d1) (Fig. 9, A

and B) (Table I). Neither control antibody anti-NCA M

(125 pmol) nor AP (30 pmol) influenced retinal growth cone morphology on either substrate (Figs. 6, E and M, 8, D and E, and 9, C and D). A similar change in growth cone morphology was observed after adding 62.5 or 125 pmol of the β1 integrin antibody JG22 (Figs. 8, J and M, and 9 C). In contrast to inhibition of the CRYPα ligand interaction, the morphological change seen with JG22 was not restricted to growth cones on BM but was also found
for growth cones growing on LN (Figs. 8, K and N, and 9 D; Table I).

Therefore, blocking the interaction of the RPTP CRYPα with its ligand from either the receptor or the ligand side, not only reduced retinal axon length but induced a significant shift in the morphology of retinal growth cones to a more filopodial appearance. This suggests that CRYPα action influences the maintenance of lamellipodia when growth cones are migrating on the intact retinal BM substrate.

The CRYPα Ligand Is Found on Glial Endfeet

The retinal basal membrane contains the endfeet of the Müller glia cells. These endfeet are visible under microscopic bright field illumination and are stained by Alexa-phalloidin (Fig. 6). Using such a staining procedure, endfeet appear as dot-like structures with a diameter of ~2 μm and with a ring-like F-actin organization. Incubating the α1-AP protein on E7 basal membranes resulted in staining of only these glial endfeet (Fig. 4 D). This suggests that the putative ligand for CRYPα is located on the glial endfeet. We tested this possibility by removing the endfeet from the retinal BM. Removal of the endfeet was performed by washing the retinal basal membrane with 2% Triton X-100 in PBS according to a published protocol (Halfter et al., 1987) and resulted in a completely transparent basal membrane (BM-Ef). In accordance with our hypothesis, there was no detectable staining of BM-Ef using the α1-AP protein (Fig. 4 E).

### Table I. Statistical Analysis of Growth Cone Morphology

| Substrate + antibody | n   | Growth cone diameter ± SEM | P     |
|----------------------|-----|---------------------------|-------|
| BM Control           | d1  | 12.86 ± 2.59              | vs. BM-Ef, <0.0001 |
|                      | d2  | 11.24 ± 2.08              | vs. BM-Ef, <0.0001 |
| BM 20 μg NCAM        | d1  | 12.22 ± 1.63              | NS    |
|                      | d2  | 10.98 ± 1.49              | NS    |
| BM 20 μg IG2         | d1  | 11.74 ± 1.36              | <0.0001 |
|                      | d2  | 5.14 ± 1.36               | <0.0001 |
| BM 20 μg β-integrin  | d1  | 7.07 ± 4.93               | <0.0001 |
|                      | d2  | 3.75 ± 2.85               | <0.0001 |
| LN Control           | d1  | 15.22 ± 2.22              | vs. BM, 0.0014 |
|                      | d2  | 13.23 ± 2.54              | vs. BM, 0.0016 |
| LN 20 μg NCAM        | d1  | 13.99 ± 2.22              | NS    |
|                      | d2  | 14.96 ± 1.95              | NS    |
| LN 20 μg IG2         | d1  | 12.95 ± 2.83              | 0.0059 |
|                      | d2  | 12.96 ± 3.31              | NS    |
| LN 20 μg β-integrin  | d1  | Not detectable            |       |
|                      | d2  | Not detectable            |       |
| BM-Ef Control        | d1  | 17.44 ± 1.96              | vs. LN, 0.0002 |
|                      | d2  | 16.41 ± 2.88              | vs. LN, <0.0001 |
| BM-Ef 20 μg NCAM     | d1  | 17.61 ± 2.94              | NS    |
|                      | d2  | 16.26 ± 2.26              | NS    |
| BM-Ef 20 μg IG2      | d1  | 15.32 ± 2.39              | 0.0163 |
|                      | d2  | 15.35 ± 2.08              | NS    |
| BM-Ef 20 μg β-integrin| d1  | 5.40 ± 5.96               | <0.0001 |
|                      | d2  | 2.59 ± 2.84               | <0.0001 |

n, Number of measured growth cones (10 growth cones from 4/6 independent experiments were analyzed); P, values from single-factor ANOVA analyzing the influence of IG2 on the same substrate and the influence of the different substrates themselves, 99% confidence interval NS indicates, no significance (statistical values > 0.05).
Washing away the endfeet demonstrated the enormous outgrowth-promoting potential of the ECM. Whereas the average axon length in controls was almost the same on LN and BM, removal of the endfeet caused an increase in the average axon length of 40% (Fig. 7 and Table II).

However, growth on BM-Ef was much less susceptible to inhibition with IG2. IG2 inhibited axon growth on complete BM by 63%, but inhibition of growth on BM-Ef was slight and similar to that seen on LN (25-30%; Fig. 7). As an additional control for specificity of inhibition by IG2 antibodies, we tested matrigel, an acellular basement membrane preparation from EHS sarcoma cells that lacks detectable CRYP ligand (McKinnell, I., and A. Stoker, unpublished work). This was considered appropriate be-

Figure 5. Quantification of the effect of IG2 and α1-AP on neurite outgrowth on LN and BM. E6 retinal explants were cultured on either LN or BM with the amounts of antibody indicated below the bars. IG2 and α1-AP reduced the average length of axon outgrowth on BM in a dose-dependent fashion (A and C). IG2 also showed a significant effect on LN (B). No effect was observed for α1-AP on LN (D). To address the possibility of an effect induced by antibody cross-linking we used anti-NCAM as a control. No effect of 20 μg anti-NCAM was observed on either LN or BM (E and F). Anti-β-integrin JG22 was used as a comparison for IG2. On BM neurite outgrowth was affected in a dose-dependent manner by JG22 (E) while on LN outgrowth was almost completely blocked by an amount of 20 μg antibody (F). One datapoint represents a measurement of one retinal strip from an independent experiment performed with at least one retinal strip. Double asterisks indicate P < 0.0001.
cause most brain BMs at stage E6 are ligand-positive, and other BMs are very difficult to isolate. Axon growth on matrigel was as prolific as on BM-Ef, and was completely unaffected by IG2 (Fig 7). Finally, anti N-CAM antibody had no influence on retinal axon growth on any substrate (Fig. 7 and Table II). These results suggest that the ligand of the PTP CRYPa is located predominantly on retinal glial endfeet structures.

On intact BMs, the anti-β1-integrin antibody (JG22) reduced outgrowth by ~70%, suggesting that, in addition to CRYPa interactions, ECM/integrin interactions are important regulators of axon growth on this tissue. As expected, inhibition of axon growth by JG22 was even greater when BM-Ef was tested, and outgrowth on LN was completely blocked. In contrast, axon growth on matrigel was inhibited only 48% by JG22. This result suggests that matrigel contains growth-promoting substances in addition to the well-characterized ECM components that are completely susceptible to inhibition by integrin antibodies (Tomaselli et al., 1986). PLL/N-cadherin mediated outgrowth was not affected by either IG2 or JG22 (Fig. 7 and Table II).

Comparison of the morphology of retinal growth cones on the three different substrates (LN, BM, and BM-Ef) revealed that growth cones on LN (Figs. 8 B and 9 D) and BM-Ef (Figs. 8 C and 9 E) are more elaborate, possessing larger d1 and d2 parameters, than growth cones on BM (Figs. 8 A and 9 C). Blocking the interaction of the RPTP CRYPa with its ligand-affected growth cone morphology on BM (Figs. 8 G and 9 C) but not on BM-Ef (Figs. 8 I and 9 E), LN (Figs. 8 H and 9 D), or matrigel (data not shown). Growth cone morphology on BM-Ef was affected by JG22.

Figure 6. The morphology of the growth cone is affected by treatment with IG2 antibody or α1-AP fusion protein in a substrate-dependent manner. Effects on morphology were seen on BM (B–D and F–H), but not on LN (J–L and N–P). On BM growth cone lamellipodia were retracted in a dose-dependent manner when adding either IG2 (A–D) or α1-AP (E–H). Arrows indicate glial endfeet structures. Bar, 0.02 mm.
Figure 7. Quantification of the influence of the growth substrate. Removal of the glial endfeet from BM resulted in strong increase in average axon length. The effect of IG2 is strongest on BM, nearly equal on BM and LN. Both effects are statistically significant (P < 0.0001). There was almost no effect of IG2 on matrigel and on N-cadherin. In the presence of glial endfeet JG2 exhibits weaker effects on axon outgrowth than on matrigel, BM-Ef, or LN. Anti-NCAM had no effect on any substrates. Double asterisks indicate P < 0.0001. See Table II for data.

Discussion

In recent years, evidence has accumulated that RPTPs play important roles in axon growth and guidance (reviewed in Chien, 1996; Desai et al., 1997a; Gershon et al., 1998; Wills et al., 1999b). Nearly all of this evidence comes from studies in invertebrates, especially from Drosophila and leech. Far less is known about the role of RPTPs in the nervous system of vertebrates. Here we present data from in vitro experiments in which we examined the role of the RPTP CRY Pα in the growth of retinal axons towards the optic fissure.

Previous experiments have shown that CRY Pα is expressed at very early stages on retinal axons and growth cones (Stoker et al., 1995a; Ledig et al., 1999). The finding that a currently unknown ligand is present on basal membranes of the eye, optic nerve, optic tract, and tectum, pointed to an important role of this receptor and its ligand in intraretinal axon growth and during growth of retinal axons towards their target, the tectum opticum (Haj et al., 1999). Blocking the CRY Pα-ligand interaction from both the receptor (IG2 antibodies) and the ligand side (α1-AP) induced dramatic changes in growth cone morphology and retinal axon length on the in vivo-like BM substrate. No effects on growth cone morphology and only weak effects on outgrowth were observed on a laminin substrate, on the physiological ECM substratum matrigel and on detergent-washed, endfeet-free basal membranes. We also observed no changes in outgrowth or growth cone morphology when we used Ig3-AP, a truncated form of CRY Pα with no ligand binding capacity (Ledig, M., unpublished work; Haj et al., 1999). Taken together with the α1-AP staining pattern on BM we suggest that the elusive CRY Pα ligand is found predominantly on the surface of endfeet of Müller glia cells. The ligand itself exerts growth promoting and growth cone lamellipodia-stimulating activities.

Table II. Statistical Analysis of the Average Axon Length

| Substrate + antibody | n | Average axon length ± SEM | P1 | P2 |
|----------------------|---|--------------------------|----|----|
| BM Control           | 19| 642.0 ± 83.4             |     |     |
| BM 20 μg NCAM        | 6 | 634.0 ± 93.7             | NS |     |
| BM 20 μg IG2         | 16| 232.4 ± 27.3             | <0.0001 | vs. BM-Ef, <0.0001 |
| BM 20 μg β-integrin  | 9 | 184.2 ± 57.0             | <0.0001 | vs. LN, 0.0001 |
| LN Control           | 14| 635.5 ± 85.5             |     |     |
| LN 20 μg NCAM        | 8 | 698.2 ± 76.1             | NS |     |
| LN 20 μg IG2         | 9 | 442.8 ± 53.1             | <0.0001 | vs. BM-Ef, <0.0001 |
| LN 20 μg β-integrin  | 5 | 20.4 ± 25.8              | <0.0001 | vs. BM-Ef, 0.0004 |
| BM-Ef Control        | 8 | 1073.6 ± 209.3           |     |     |
| BM-Ef 20 μg NCAM     | 8 | 1014.8 ± 119.4           | NS |     |
| BM-Ef 20 μg IG2      | 13| 796.6 ± 94.3             | 0.0015 | vs. BM, <0.0001 |
| BM-Ef 20 μg β-integrin | 6 | 220.6 ± 69.7             | <0.0001 | vs. BM, NS |
| MG Control           | 9 | 970.4 ± 173.3            |     |     |
| MG 20 μg NCAM        | 3 | 883.8 ± 65.7             | NS |     |
| MG 20 μg IG2         | 11| 909.8 ± 109.7            | NS |     |
| MG 20 μg β-integrin  | 7 | 470.5 ± 74.4             |     |     |
| NC/PLL Control       | 9 | 281.5 ± 35.9             |     |     |
| NC/PLL 20 μg NCAM    | 3 | 256.8 ± 30.9             | NS |     |
| NC/PLL 20 μg IG2     | 6 | 252.7 ± 41.3             | NS |     |
| NC/PLL 20 μg β-integrin | 3 | 213.4 ± 5.8              | 0.0014 | vs. BM-Ef, 0.001 |

n, Number of independent experiments with at least two retinal strips; P1, values from single-factor ANOVA (ANOVA was used because most of the data show a normal distribution) analyzing the influence of different antibodies on the same substrate, 99% confidence interval; P2, values from single-factor ANOVA analyzing the influence of the growth substrate when treated with the same antibody, 99% confidence interval (the substrate that was used for the comparison is indicated); NS indicates no significance (statistical values > 0.05).
We have demonstrated that the glial endfeet of the retinal BM contain a balance of positive and negative cues. For example, removal of the glial endfeet leads to an enormous increase in axon outgrowth on BMs, whereas only on intact BMs do antibodies to CRYPα and the CRYPα1-AP fusion protein induce dramatic reductions in outgrowth. Previous data have demonstrated that the net outcome of these factors is indeed permissive for retinal axon growth (Stier and Schlosshauer, 1999). In the presence of these endfeet, the disturbance of CRYPα-ligand interactions causes a strong decrease of axon outgrowth. This suggests that during normal, intraretinal axon outgrowth, the growth-facilitating or -promoting role of CRYPα is in constant balance with negative influences on the growth cone. Therefore, this work places the RPTPs in a critical position in the regulation of intra-growth cone phosphotyrosine levels and maintenance of axon outgrowth (Cummings, 1999).

Glial endfeet are present on retinal basal membranes (Bauch et al., 1998), but their molecular components are not completely characterized. The laminin/hidrogen complex is present in the BM (Cohen et al., 1987). It was recently shown that this complex binds in vitro to the fifth fibronectin type III domain of the long isoform of LAR.
(O'Grady et al., 1998), an RPTP structurally related to CRYPα2. However, this isoform of LAR is not expressed in the nervous system. We would not expect a similar interaction of CRYPα1 with laminin, as in vitro binding assays failed to detect an interaction of CRYPα1 or CRYPα2 with laminin 1 or 2 (Haj et al., 1999; McKinnell, I.W., and A.W. Stoker, unpublished work). In the same in vitro assays, matrigel did not bind to CRYPα1. Recent work also suggests that CRYPα does not interact homophilically (Haj et al., 1999). These data underline our idea that the CRYPα ligand is a currently unknown transmembrane, membrane-anchored, or membrane-asso-
The glial endfeet, therefore, appear to have a strict growth controlling function containing a balance of growth promoting and inhibiting factors on their surfaces.

The data also indicate that both integrins and CRYP\(\alpha\) can both influence lamellipodial dynamics, either independently or synergistically. What could be the link between CRYP\(\alpha\) and lamellipodia?

Well-known molecular regulators of lamellipodia and filopodia in fibroblasts are the small GTP-binding proteins of the Rho family, Rac and Cdc42, respectively (Nobes and Hall, 1995, 1999; Hall 1998). A valuable evidence suggests that they exert similar functions in neuronal growth cones (reviewed in Mueller, 1999). Control of the growth cone actin cytoskeleton by RPTPs could be achieved by interacting adapter proteins, like Trio. Trio binds to the intracellular domain of the CRYP\(\alpha\) family member, LAR, and has two guanine exchange factor domains specific for Rac and Rho (Debant et al., 1996; Bellanger et al., 1998). Furthermore, the Caenorhabditis elegans Trio homologue, UNC-73, was shown to be required for axon growth and guidance (Steven et al., 1998). The phenotypic similarities of null mutants for DLAR and the Drosophila R ac suggest their interaction in guidance of intersegmental nerve b (Kaufmann et al., 1998). Further evidence for an important function of RPTPs in controlling the actin cytoskeleton comes from two recent papers showing that profilin is regulated by DLAR involving the cytoplasmic tyrosine kinase abl and its substrate enabled (Wills et al., 1999a,b). Trio could also provide a connection between DLAR and profilin via Rho and phosphatidylinositol 4-phosphate 5-kinase (Chong et al., 1994).

From our data that blocking of CRYP\(\alpha\)-ligand interactions on BM leads to changes in the actin cytoskeleton and lamellipodia formation, it seems most likely that the CRYP\(\alpha\) downstream signaling pathway also involves Rac. This may be related to a similar process as shown for integrins (Keely et al., 1997; Clark et al., 1998; Kuhn et al., 1998) (Fig. 10). There is likely to be considerable cross-talk between RPTPs and other extracellular receptors. This is especially important for recognition of multiligand complexes consisting of cell adhesion molecules and other growth cone guidance activities (Holland et al., 1998). The downstream signaling pathways of both CRYP\(\alpha\) and integrin receptors seem to converge as well. There are several examples for an interplay of RPTPs and integrins (Shenoi et al., 1999; Su et al., 1999), and it is quite likely that the same is true for CRYP\(\alpha\). The LAR-interacting protein (LIP) localizes type II RPTPs to focal adhesions (Serrapages et al., 1995, 1998; Pulido et al., 1995), and, therefore, brings them into the close neighborhood of integrins. An interplay between RPTPs and integrin could then happen on the level of the small GTPases and Src-family of cytoplasmic kinases (Schlaepfer and Hunter, 1998), both of which are shared in downstream pathways. This could result in the joint control of adhesive and deadhesive properties of cells or growth cones (Helmke et al., 1998). Such a relationship has been shown, for example, with the T and B cell RPTP CD45 and integrins (Roach et al., 1997; Shenoi et al., 1999).
Based on the data we have presented, we can formulate a basic model for some of the signaling processes downstream of CRYP and integrins in retinal growth cones (Fig. 10). It is clear that laminin-integrin interactions alone can stimulate growth cone migration and lamellipodia formation in the absence of other incoming signals (Tomaselli et al., 1996). On a complete BM, however, we suggest there is a positive, CRYP-dependent signal and a balancing negative signal(s) coming from an independent ligand-receptor complex. The latter signal could partly suppress integrin-promoted outgrowth and lamellipodia formation, by downregulating, directly or indirectly, the Rac pathway. This is counteracted by the positive CRYP signal. Thus, loss of CRYP signal in our experiments induces a significant block in neurite outgrowth. The modest influence of the Ig2 antibody on a pure laminin substrate could be partly explained by a low level of activity in both the CRYP and negative signals, in the absence of their cognate ligands. CRYP antibodies would then cause a only slight suppression of integrin-mediated outgrowth, without grossly altering lamellipodia. This model needs to be tested further, but possible candidates for the negative signals would include nongraded E ph receptors, and their ephrin ligand, which are known to be present on glial end-feet (Braisted et al., 1997). A nother interesting possibility could be that there are some cis-interactions between integrins and CRYP on the growth cone.

PTP μ, another membrane tyrosine phosphatase, was recently shown to mediate homophilic trans-interactions (Gebbink et al., 1995; Zondag et al., 1995) and to be involved in retinal axon growth (Burden-Gulley and Brady-Kalnay, 1999). PTP μ interacts with three different, calcium-dependent cell adhesion molecules, N- pc - and E-cadherin (Brady-Kalnay et al., 1998; Burden-Gulley and Brady-Kalnay, 1999). It was shown that N- and R-cadherin promote outgrowth of neurites (e.g., Bixby and Zhang, 1990; Edies and Takeichi, 1993). U sed as a substrate, PTP μ stimulated neurite outgrowth of E8 chick RGC neurites and on an N-cadherin substrate; downregulation of this phosphatase resulted in a significant decrease of the retinal neurite length, suggesting that the phosphatase activity of PTP μ is important for growth of RGC neurites on N-cadherin (Burden-Gulley and Brady-Kalnay, 1999).

R PTP β/PTP γ is another outgrowth-inducing R PTP (Peles et al., 1995; Sakurai et al., 1997). It binds to other members of cell surface recognition complexes, among them contactin/F11 (Brümmerendorf et al., 1998) and Nr-CAM, enabling signal transduction into the growth cone (Peles et al., 1998). But the relevance of this R PTP for the development of the retinotectal system remains to be elucidated.

During formation of the retinotectal map, there now appear to be, besides the integrins and N-cadherin, at least two different R PTPs involved in outgrowth of retinal ganglion cell axons: CRYP and its yet undefined ligand, and PTP μ. Our results suggest that besides the RTKs (Drescher et al., 1997; Muehler et al., 1996), R PTPs such as CRYP play an extremely important, complementary role in promoting formation of the visual system. It remains to be seen if gene deletion of R PTPs such as the mammalian orthologue of CRYP, R PTP Δ (Elchebly et al., 1999; Wallace et al., 1999), results in disturbance of the retinotectal projection.

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