PTPμ Regulates N-Cadherin–dependent Neurite Outgrowth

Susan M. Burden-Gulley and Susann M. Brady-Kalnay

Department of Molecular Biology and Microbiology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106-4960

Abstract. Cell adhesion is critical to the establishment of proper connections in the nervous system. Some receptor-type protein tyrosine phosphatases (RPTPs) have adhesion molecule–like extracellular segments with intracellular tyrosine phosphatase domains that may transduce signals in response to adhesion. PTPμ is a RPTP that mediates cell aggregation and is expressed at high levels in the nervous system. In this study, we demonstrate that PTPμ promotes neurite outgrowth of retinal ganglion cells when used as a culture substrate. In addition, PTPμ was found in a complex with N-cadherin in retinal cells. To determine the physiological significance of the association between PTPμ and N-cadherin, the expression level and enzymatic activity of PTPμ were perturbed in retinal explant cultures. Downregulation of PTPμ expression through antisense techniques resulted in a significant decrease in neurite outgrowth on an N-cadherin substrate, whereas there was no effect on laminin or L1-dependent neurite outgrowth. The overexpression of a catalytically inactive form of PTPμ significantly decreased neurite outgrowth on N-cadherin. These data indicate that PTPμ specifically regulates signals required for neurites to extend on an N-cadherin substrate, implicating reversible tyrosine phosphorylation in the control of N-cadherin function. Together, these results suggest that PTPμ plays a dual role in the regulation of neurite outgrowth.

Key words: neurite outgrowth • protein tyrosine phosphatase • cadherin • cell adhesion • retina

THE development of the nervous system is a complex process requiring the formation of a large number of specific connections by a variety of neuronal cell types and target tissues. Some of the factors that regulate axonal pathfinding include contact-mediated growth promoting or repulsive molecules, as well as diffusible attractant and repulsive factors (reviewed by Tessier-Lavigne and Goodman, 1996). Contact-dependent guidance is thought to be mediated by a series of adhesive events between the growth cone, the specialized region at the distal tip of a growing axon, and molecules expressed on other cells or in matrices that form the "substrate" for migration. Presumably, the growth cone responds to these cues by initiation of a local signal that ultimately regulates the direction of axonal extension. The role of adhesion molecules in contact-dependent guidance has been extensively studied, yet the signaling events involved in this process are not well understood. The present study investigates the role of a unique cell adhesion molecule, the receptor-type protein tyrosine phosphatase mu (PTPμ),[1] in contact-dependent signaling and neurite outgrowth. PTPμ is expressed in several regions of the central nervous system during development and is capable of mediating adhesion, which suggests it could be one of the adhesion molecules that plays a role in neurite outgrowth.

Due to the complexity of the developing nervous system, many studies, including the one described here, have used in vitro model systems to gain a better understanding of the mechanisms that regulate axonal growth. A number of nervous system–derived cell surface proteins have been isolated, but only a subset of these have been shown to promote neurite outgrowth when used as a substrate in vitro. These proteins are subdivided into three major classes: the integrins and their ligands the extracellular matrix molecules (ECM), cadherins, and the immunoglobulin superfamily of cell adhesion molecules (CAMs). Neuronal cells express several integrin receptors and will extend neurites on a subset of ECM molecules including laminin, vitronectin, thrombospondin, janusin, and tenascin (Reichardt et al., 1990; Neugebauer et al., 1991; Reichardt and Tomaselli, 1991; Lochter and Schachner, 1993; Culp et al., 1997). The cadherins are calcium-dependent adhesion molecules and only N- and R-cadherin have been shown to promote neurite outgrowth (Bixby and Zhang, 1990; Redies and Takeichi, 1993). In addition, some Ig superfamily molecules mediate cell–cell adhesion and have also been shown to promote neurite outgrowth (Brummendorf and Rathjen, 1993; Burden-Gulley and Lem-

Address correspondence to Susann Brady-Kalnay, Department of Molecular Biology and Microbiology, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4960. Tel.: (216) 368-0330. Fax: (216) 368-3055. E-mail: smb4@po.cwru.edu

1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; GFP, green fluorescent protein; RGC, retinal ganglion cell; RPTP, receptor-type protein tyrosine phosphatase.

mon, 1995). L1 and NCAM are two examples of Ig superfamily CAMs that are abundantly expressed in the developing and adult nervous systems, and are capable of promoting neurite outgrowth (reviewed in Burden-Gulley and Lemmon, 1995; Brummendorf and Rathjen, 1998).

A number of receptor protein tyrosine phosphatases (RPTPs) resemble Ig superfamily CAMs such as NCAM, and appear to directly mediate adhesion or associate with other adhesion molecules (reviewed in Brady-Kalnay and Tonks, 1995; Streuli, 1996; Neel and Tonks, 1997; Brady-Kalnay, 1998). The coupling of CAM-like extracellular domains and phosphatase enzymatic activity within one molecule suggests that changes in the adhesive state of the phosphatase could potentially alter enzymatic activity. One such RPTP, PTPμ, is characterized by an extracellular segment that contains one MAM domain (meprins, A5, mu), one Ig domain, and four fibronectin type III repeats (Gebbink et al., 1991). Expression of PTPμ induced the aggregation of nonadhesive cells (Brady-Kalnay et al., 1993; Gebbink et al., 1993), and both the MAM domain (Zondag et al., 1995) and the Ig domain (Brady-Kalnay and Tonks, 1994a) have been shown to play a role in PTPμ-mediated cell–cell adhesion. Together, these studies demonstrated that the binding is homophilic (i.e., the "ligand" for PTPμ is an identical PTPμ molecule on an adjacent cell).

The juxtamembrane domain of PTPμ has 20% amino acid identity to the conserved intracellular domain of the cadherins (Brady-Kalnay and Tonks, 1994b). The cytoplasmic segment of cadherins binds to catenins that link this complex to the actin cytoskeleton (Gumbiner, 1995; Aberle et al., 1996). Previously, we showed that PTPμ associates with the cadherin/catenin complex (Brady-Kalnay et al., 1995; Hiscox and Jiang, 1998), specifically with N-, E-, and R-cadherins (also called cadherin-4; Brady-Kalnay et al., 1998). In addition, the intracellular segment of PTPμ was demonstrated to bind directly to the intracellular domain of E-cadherin (Brady-Kalnay et al., 1995), and the COOH-terminal 38 amino acids of E-cadherin were required for this interaction (Brady-Kalnay et al., 1998). However, a role for PTPμ in the regulation of cadherin function has not yet been shown.

The PTPμ protein is abundant in many parts of the central nervous system (Gebbink et al., 1991; Brady-Kalnay et al., 1995; Sommer et al., 1997; Brady-Kalnay, 1998) and is developmentally regulated in the retina. The retina is one of the best characterized and experimentally tractable systems for studying both cell–cell adhesion and development. The retina is comprised of a number of different cell types, and the molecular interactions of multiple CAMs are known to regulate retinal histogenesis and axonal pathfinding (Silver and Rutishauser, 1984; Matsunaga et al., 1988a,b; Brittis et al., 1995; Brittis and Silver, 1995). In the retina, PTPμ is primarily expressed on retinal ganglion cells (RGCs) whose sole function is to communicate via their neuronal processes with the brain. RGC axons migrate along the surfaces of neuronal and glial cells in the brain during development, thus using the CAMs on the surfaces of these cells as a substrate for neuronal migration.

Due to the homophilic binding nature of PTPμ, its developmental pattern of expression in the retina, and interaction with cadherins in other tissues, we tested the hypothesis that PTPμ regulates neurite outgrowth. In this report, we used an in vitro retina explant model system to study neurite outgrowth. We provide evidence that PTPμ is capable of promoting neurite outgrowth and cell migration when used as a culture substrate. The neurite outgrowth activity was specific to PTPμ since antibodies against PTPμ completely inhibited outgrowth on a PTPμ substrate. In addition, PTPμ was found in a complex with N-cadherin in retinal tissues and RGC neurites. To determine the physiological significance of an association between PTPμ and N-cadherin, the expression level and enzymatic activity of PTPμ were perturbed in retinal explants. Downregulation of PTPμ expression through antisense techniques resulted in a significant decrease in neurite outgrowth on an N-cadherin substrate, whereas there was no effect on laminin or L1-dependent neurite outgrowth. The overexpression of a catalytically inactive form of PTPμ significantly decreased neurite outgrowth on N-cadherin, thus illustrating the importance of the enzymatic activity of the PTPμ phosphatase in this process. These data indicate that PTPμ specifically regulates signals required for neurites to extend on an N-cadherin substrate, implicating reversible tyrosine phosphorylation in the control of N-cadherin function. Together, these results suggest that PTPμ plays a dual role in the regulation of neurite outgrowth during retinal development.

Materials and Methods

Antibodies and Reagents

Monoclonal antibodies against the intracellular (SK15, SK18) and extracellular (BK2, BK9) domains of PTPμ have been described (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994a). The MB4 monoclonal antibody was generated against the peptide CSH 338 (Brady-Kalnay and Tonks, 1994a) from the immunoglobulin domain of PTPμ (amino acids 231–256). The 494 polyclonal antibody generated against a PTPμ peptide (amino acids 42–60) has been described (Brady-Kalnay and Tonks, 1994a). A monoclonal pan-cadherin antibody (SMP) generated against the COOH terminus of chick N-cadherin was obtained from Sigma Chemical Co. The NCD-2 monoclonal antibody was generously provided by Dr. Gerald Grunwald (Thomas Jefferson University, Philadelphia, PA) from hybridoma cells generated by Dr. Masotoshi Takeichi (Hatta and Takeichi, 1986). A polyclonal antibody against N-cadherin (7873) was kindly provided by Dr. John Hemperly (Becton Dickinson Labs, Research Triangle Park, NC). Antibodies against chick NCAM (5e, RO28 and RO32) and L1 (RO21) were generously provided by Dr. Urs Rutishauser (Case Western Reserve University). Monoclonal antibodies against chick L1 (8D9), a bipolar neuron-specific antigen (3G3), and a Müller glia-specific antigen (5A7) were generously provided by Dr. Vance Lemmon (Case Western Reserve University). RPMI 1640 medium, laminin, and fetal bovine serum were obtained from GIBCO BRL. Aprotinin and leupeptin were obtained from Boehringer Mannheim Biochemicals. Tween-20 was obtained from Fisher Scientific Co. All other reagents were obtained from Sigma Chemical Co.

Purification of PTPμ from Brain

Adult rat brains were minced and homogenized (Bellco Biotechnology) in a solution of 0.32 M sucrose in 50 mM Tris-HCl, 150 mM NaCl (TBS), pH 8.0, containing protease inhibitors 5 mM EDTA, 10 μg/ml turkey trypsin inhibitor, 2 mM benzamidine hydrochloride, and 200 μM phenylmethylsulfonylfluoride. The homogenate was layered onto a 0.8 M, 1.2 M sucrose gradient and centrifuged at 25,000 rpm for 45 min (SW28 rotor; Beckman Instruments, Inc.). The membrane layer was diluted with TBS and respun at 50,000 rpm for 30 min (Ti 70.1 rotor; Beckman Instruments, Inc.). The pellet was then extracted with 1% sodium deoxycholate in 50 mM Tris-HCl, pH 8.0. The membrane extract was respun at 50,000 rpm for 30 min

and the supernatant was incubated overnight at 4°C with CNBr Sepharose 4B beads (Pharmacia LKB Biotechnology) that had been covalently coupled with the SK15 PTPμ antibody. The beads were washed extensively with 50 mM Tris-HCl containing 0.5% sodium deoxycholate and 0.5% NP-40, pH 8.0, followed by 10 mM Tris-HCl, pH 8.0. The protein was eluted from the column with 0.1 M diethylamine, pH 11.5, and neutralized with 2 M Tris-HCl, pH 3.6. For SDS-PAGE, sample buffer (4% SDS, 20% glycerol, 0.2 M dithiothreitol, bromphenol blue, 0.12 M Tris, pH 6.8) was added and boiled for 5 min at 95°C. L1 and N-cadherin were purified from chick brains using previously described procedures (Lemmon et al., 1989; Bixby and Zhang, 1990).

Protein purity was checked by two different methods. First, the eluted protein fractions were separated by 4–15% SDS-PAGE (gradient Tris-HCl gels; Bio-Rad Laboratories) and the gel was silver stained. In a second procedure, the eluted fractions were immunoblotted as previously described (Brady-Kalnay et al., 1995).

Dish Preparation and Culture of Retinal Explants and Dissociated Cells

35-mm tissue culture dishes (Falcon Labware) were coated with nitrocellulose in methanol (Lagenaur and Lemmon, 1987) and allowed to dry. A small amount of protein (2–4 μg) was spread across the center of the dishes, and they were incubated 30 min at room temperature. Remaining binding sites on the nitrocellulose were blocked with 2% BSA in PBS, and the dishes were rinsed with RPMI-1640 medium.

Retinal explant cultures were made according to a previously described procedure (Halfter et al., 1983; Drazba and Lemmon, 1990). In brief, embryonic day 8 (stage 32.5–33 according to Hamburger and Hamilton, 1951) White Leghorn chick eyes were dissected and the retina was flattened with the photoreceptor side down onto black nitrocellulose filters (0.45-μm pore size; Vanguard International, Inc.) that had previously been incubated in 0.05% concanavalin A to enhance attachment of the retina to the filter. The filter was then cut into 350-μm wide strips perpendicular to the optic fissure using a McIlwain tissue chopper. Strips were inverted onto substrate-coated culture dishes so that the ganglion cell layer was directly adjacent to the substratum. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum/2% chick serum/penicillin-streptomycin-fungizone and incubated at 37°C in 95% air/5% CO_2.

Neurite outgrowth on PTPμ, N-cadherin, and L1 substrates was inhibited by the addition of culture supernatant from the BK2 (anti–PTPμ), NCD2 (anti–N-cadherin), and 8D9 (anti–L1) hybridoma cells, respectively, into the medium at the time of plating retinal explants as described above. The medium was supplemented with fetal bovine serum and chick serum to maintain equivalent levels of growth factors to controls. Neurite outgrowth was examined at 24, 48, and 72 h after plating.

Dissociated retina cultures were prepared from E6 embryos using the procedure above, except that the retinas were incubated in 0.25% trypsin and 0.1% EDTA (Mediatech Cellgro) for 20 min at 37°C. The retinas were dissociated by trituration and resuspended in RPMI-1640/10% tryptose phosphate broth/4% fetal bovine serum/1% chick serum/gentamycin, and then cultured in 12-well plates (Falcon Labware). E6 retinas were used because a large percentage of the cells are still mitotic at this age, which is an important requirement for retroviral-mediated gene transfer. Expression of the exogenous gene was repressed by culturing infected cells in the presence of 3 μg/ml tetracycline (see below).

Immunolabeling of Retina Sections and Cultured Explants

For immunohistochemical labeling of retina sections, eyes from E8 chicks were fixed with 4% paraformaldehyde, 0.01% glutaraldehyde in PEM buffer (80 mM Pipes, 5 mM EGTA, 1 mM $MgCl_2$, 3% sucrose), pH 7.4. The tissue was rinsed and cryoprotected with 20% sucrose in PBS and frozen in OCT medium (EM Sciences). Cryostat sections were cut at 7-μm intervals, adhered to gelatin coated slides, and stored at −20°C. Sections were permeabilized and blocked with 1% Triton, 20% goat serum in PBS, and then incubated overnight at 4°C with primary antibodies diluted into block buffer (20% goat serum, 1% BSA, 0.5% saponin in PBS). After extensive rinsing, sections were incubated with fluorescein-conjugated secondary antibodies, rinsed extensively with PBS, and then coverslipped with IFF mounting medium (0.5 M Tris-HCl, pH 8.0, containing 20% glycerol and 1 mg/ml *p*-phenylenediamine). Immunolabeled sections were examined using a 40× objective on an Axiophot microscope (Carl Zeiss, Inc.), and images captured using a Hamamatsu-cooled CCD camera.

For immunocytochemical labeling of retinal explant cultures, the cultures were fixed as above, and then rinsed with PBS and incubated with block buffer. Cultures were incubated with primary antibodies in block buffer overnight at 4°C. After rinsing with PBS, the cultures were blocked with TNB reagent (supplied in TSA-direct kit; NEN Life Science Products) for 30 min, and then incubated with fluorophore or HRP-conjugated secondary antibodies diluted in TNB for 1 h at room temperature. After extensive rinsing with TNT buffer (0.1 M Tris, 0.15 M NaCl, 0.05% Tween-20), the cells were incubated with the tyramide-FITC reagent in 1× amplification diluent for 10 min to deposit FITC onto the HRP-conjugated secondary antibodies. After TNT rinses, the cultures were coverslipped with IFF mounting medium and examined using a 40× objective on a microscope (405M; Carl Zeiss, Inc.).

Antibody cross-linking ("patching") experiments were done by incubating live retinal cultures with a polyclonal antibody against the extracellular domain of PTPμ (494) for 40 min at 37°C. The cultures were then rinsed, fixed, and permeabilized as above. The cells were processed for immunocytochemistry as above using a monoclonal antibody against N-cadherin (NCD2) or NCAM (5e) and the appropriate secondary antibodies. The double-labeled samples were examined using a 100× Neofluar objective (1.3 numerical aperture) on a confocal microscope (LSM-410; Carl Zeiss, Inc.).

Immunoprecipitations

Antibodies were covalently coupled to CNBr Sepharose 4B (Pharmacia LKB Biotechnology) using the manufacturer's protocol, or Protein A beads using a previously described protocol (Brady-Kalnay et al., 1995). E8 retinas were homogenized with a tissue tearor (200; PRO Scientific Inc.) in lysis buffer (1% Triton, 20 mM Tris, pH 7.6, 2 mM $CaCl_2$, 150 mM NaCl, 1 mM benzamidine hydrochloride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM sodium orthovanadate, 0.1 mM ammonium molybdate, 0.2 mM phenyl arsine oxide), and incubated on ice for 45 min. Triton-insoluble material was removed by centrifugation at 14,000 rpm, and the lysate was incubated with antibody-coupled beads overnight at 4°C. The beads were washed extensively with lysis buffer, and then boiled in sample buffer and separated by 6% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and immunoblotted as described (Brady-Kalnay et al., 1995).

Construction and Expression of the PTPμ Retroviruses

The retroviral system used is a tetracycline-repressible promoter-based ("tet-off") system (Paulus et al., 1996). Using the pBPSTR1 vector generously provided by Dr. Steven Reeves (Harvard Medical School, Charlestown, MA), the following constructs were generated: antisense PTPμ, sense PTPμ, and a c → s mutant form of PTPμ. A PTPμ antisense plasmid was generated that contained PTPμ coding sequence (base pairs 2449–4358) in the opposite orientation to the promoter. The sense plasmid contained almost the entire coding sequence of PTPμ (base pairs 1–4350; i.e., it only lacked the last two amino acids and the stop codon). This was done to create an in-frame fusion with the green fluorescence protein (GFP) at the COOH terminus that we call PTPμGFP. This plasmid was generated by digesting full length PTPμ in bluescript (Brady-Kalnay et al., 1993) and ligating it into pEGFP-N3 from Clonetech. The mutant form of PTPμ containing the cysteine-to-serine mutation at residue 1095 was generated by PCR, and a BglII/BspE1 fragment was subcloned to replace that same fragment in the wild-type form of PTPμ in the vector PTPμ/pEGFP-N3. The resulting plasmid, c → s mutant PTPμGFP, was sequenced to confirm that the single amino acid mutation was present. The c → s mutation renders the phosphatase catalytically inactive (Barford et al., 1994). The pEGFP plasmids containing either wild-type or mutant PTPμ were subcloned into the tetracycline-regulatable retroviral vector, pBPSTR1. A replication-defective amphotrophic retrovirus was made by transfecting the PA317 helper cell line (CRL-9078; American Type Culture Collection) with the respective PTPμ-containing plasmids. Control virus was generated by transfecting PA317 helper cells with the pBPSTR1 plasmid alone. The virus was added to cells in the presence or absence of 3 μg/ml tetracycline. When tetracycline was added, expression of the viral gene was inhibited. Reduction in endogenous PTPμ expression was verified by immunoblotting lysates from infected cells with antibodies to PTPμ. The density of the bands on the immunoblot films was measured using the MetaMorph image analysis program (Universal Imaging Corp.).

Infection of Retinal Explants with Retrovirus

Retroviral-mediated gene transfer requires that the infected cells are still mitotic in order to incorporate and express the retroviral gene. RGC neu-

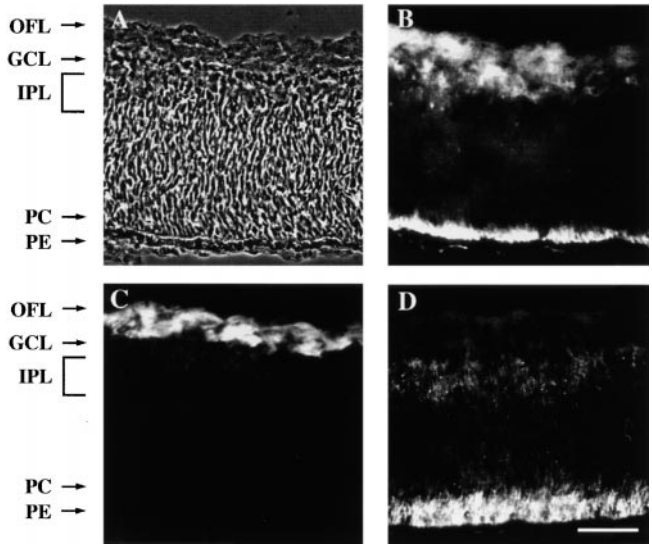

Figure 1. Expression of PTPμ in chick retina. E8 chick retina sections were immunohistochemically labeled with antibodies against (B) PTPμ, (C) L1, or (D) a bipolar neuron-specific antigen. (B) PTPμ labeling was present predominantly in regions that labeled positively for retinal ganglion cells (C) and bipolar neurons (D). Phase contrast image is shown in A. OFL, optic fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; PC, precursor cells; PE, pigmented epithelium. Scale bar, 50 μm.

rons begin to drop out of cell division at E2–3 at a region just dorsotemporal to the optic fissure, and the wave of maturation continues outward from this region in a spatiotemporal fashion (Halfter et al., 1983; Prada et al., 1991). To infect the greatest number of cells for these experiments, retinas from viral-free E3.5–4 chicks (stage 20–23) (Spafas Inc.) were used. At this age, robust neurite outgrowth occurred on N-cadherin, laminin, and L1 substrates, but not on PTPμ. Therefore, PTPμ could not be used as a test substrate for these experiments. The dissection and plating procedure is as described above except that the retinas were cut at 250-μm intervals. Explant strips from each retina were laid in alternating fashion onto two similar substrate-coated dishes, with four explants per dish, then 28 μg of polybrene and 1 ml of virus-containing medium were added for a 6–18-h incubation at 37°C. After incubation in virus, the medium was exchanged with normal culture medium. Cultures were examined at ~24 and 48 h after plating, and neurite outgrowth from each explant was photographed.

Quantitation of Neurite Outgrowth

To quantify the neurite outgrowth, the 35-mm negatives were scanned and the digitized images were analyzed using the MetaMorph image analysis program. Lengths of the five longest neurites per explant were measured perpendicular to the explant tissue. To measure the number of neurites per explant, the region of neurite outgrowth was outlined to define the region of interest, the neurites were highlighted using the threshold function, and the total number of highlighted pixels per region of interest was calculated. This method provided a means to compare density between control and test conditions on each substrate. The neurite length and density measurements were analyzed by Fisher's PLSD, Scheffe, and Student's *t* test (Statview 4.51; Abacus Concepts, Inc.), and similar results were obtained with each of these tests for each experiment. The data from all like experiments were combined and plotted (Cricketgraph III; Computer Associates International, Inc.).

Results

Expression of PTPμ in the Retina

During development, the retina becomes laminated such that cells of a particular type localize to distinct regions within the adult retina. A phase contrast image of an embryonic day 8 (E8) chick retina cross-section illustrates the limits of the neural retina and incomplete lamination at this stage of development (Fig. 1 A). Immunohistochemical labeling of E8 retina sections showed that the greatest level of PTPμ expression was present in the retinal ganglion cell layer and in their processes that make up the optic fiber layer (Fig. 1 B). RGC neurons were verified in this region by labeling with an antibody against chick L1 that specifically recognizes the RGC axons (Fig. 1 C).

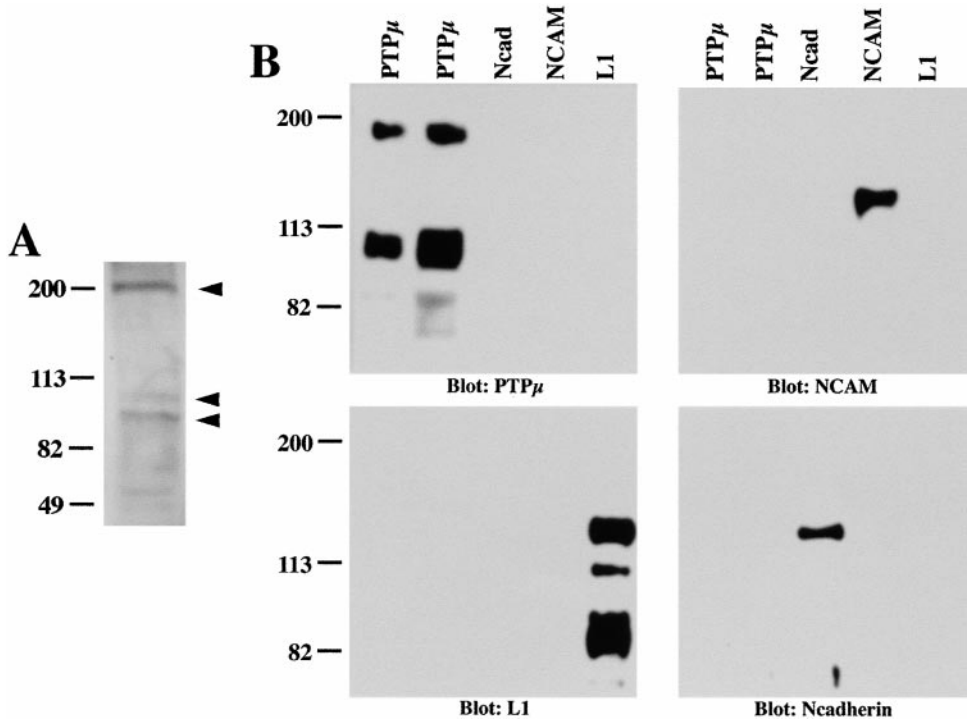

Figure 2. Purification of PTPμ from brain. PTPμ was purified from adult rat brain by immunoaffinity methods. (A) The eluted PTPμ protein was separated by 5–15% gradient SDS-PAGE and silver stained. Both the full-length protein (200 kD) and the proteolytically processed extracellular (105 kD) and intracellular (100 kD) fragments were detected (arrows). (B) The purified PTPμ protein was examined by immunoblotting to confirm that the preparation was not contaminated by other cell–cell adhesion molecules that promote neurite outgrowth. N-cadherin, NCAM, L1, and two different preparations of PTPμ were purified and equal amounts of the proteins were separated by 6% SDS-PAGE. Immunoblots using antibodies to PTPμ (top left), NCAM (top right), L1 (bottom left), or N-cadherin (bottom right) are shown.

PTPμ was also expressed at high levels in a region directly adjacent to the pigmented epithelium, which is the outer limit of the neural retina and is thought to be populated in part by mitotic precursor cells (Fig. 1 B) (Mey and Thanos, 1992). In addition, PTPμ was observed in a region of the retina that labeled positively with the 3G3 antibody against bipolar neurons (Fig. 1 D). This immunolabeling pattern is consistent with that previously described for bipolar neurons (Tsui et al., 1992; Mi et al., 1998). Since the axons of RGCs form the optic nerve and are the sole output from the retina to the brain, the high expression of PTPμ on these cells is consistent with a putative role for PTPμ in neurite outgrowth.

Purification of PTPμ

To determine whether PTPμ was capable of promoting neurite outgrowth, it was purified from brain by immunoaffinity methods using a monoclonal antibody against the intracellular domain of the protein (SK15). The PTPμ purification strategy included stringent detergent washes of the column before elution to exclude any associated proteins. The full length PTPμ protein from brain was ~200 kD, and two fragments of ~105 kD (predominantly extracellular form) and 100 kD (the intracellular domain, transmembrane region, and a short stretch of the extracellular domain) were observed (Fig. 2 A, arrows). These fragments have been shown to be due to normal proteolytic processing of the protein into noncovalently associated extracellular and intracellular fragments, respectively (Brady-Kalnay and Tonks, 1994a). The eluted PTPμ protein was examined by immunoblotting to confirm that the preparation was not contaminated by other CAMs that promote neurite outgrowth (Fig. 2 B). The samples included purified proteins from brain: PTPμ (two different preparations), N-cadherin, NCAM, and L1. N-cadherin, NCAM, and L1 were detected in the appropriate purified protein lanes, but were absent from the PTPμ preparations. PTPμ was only detected in the PTPμ preparations. These results clearly show that the PTPμ purified using these stringent conditions was not contaminated with N-cadherin, L1, or NCAM, three CAMs that are highly expressed in brain and have been previously demonstrated to promote neurite outgrowth (Rutishauser, 1983; Lagenaur and Lemmon, 1987; Bixby and Zhang, 1990).

PTPμ Promotes Neurite Outgrowth

Retinal ganglion cell axons migrate along the surface of neuronal or glial cells in the brain during development (Mey and Thanos, 1992); therefore, CAMs expressed on the surface of these cells can serve as a substrate for neuronal migration. Due to the complexity of the developing brain, we have used an in vitro model system to gain a better understanding of the mechanisms that regulate axonal growth. The in vitro system uses a purified protein-coated dish as a substrate and measures the ability of neuronal cells to extend neurites on that substrate. The homophilic binding activity of PTPμ and expression by RGC neurons led us to test whether RGC neurons were capable of extending neurites on a PTPμ substrate. The purified PTPμ or N-cadherin preparations from brain (Fig. 2) were individually coated as a substrate on dishes (Lagenaur and Lemmon, 1987) and used to culture E8 chick retinal explants (Fig. 3). Neurite outgrowth on a PTPμ substrate was observed within 2 d of plating, and the length and density of outgrowth increased over the following 2 d. Neurites grew out from explants derived from E6, 8, and 10 chick retinas, but not from E4 (data not shown), suggesting that PTPμ-dependent neurite outgrowth may be developmentally regulated. Alternatively, since the expression of PTPμ is very low at E4 (see Fig. 6), we may be unable to detect neurite outgrowth in vitro at that stage. The most robust outgrowth occurred from E8 retinas and this age was used for the experiments described here. On a PTPμ substrate, the neurites tended to be somewhat fasciculated (Fig. 3, D and E). In addition, the growth cones were small and spiky in nature, with multiple long filopodial processes and a small lamellipodial region (Fig. 3 F). In contrast, outgrowth on N-cadherin was more robust and was observed within 1 d of culture, suggesting a faster growth rate on N-cadherin than on PTPμ. Additionally, the neurites were less fasciculated on N-cadherin (Fig. 3, A and B), and the growth cones possessed a much larger lamellipodial area and several short filopodial processes (C). The lower density of neurite outgrowth on the PTPμ

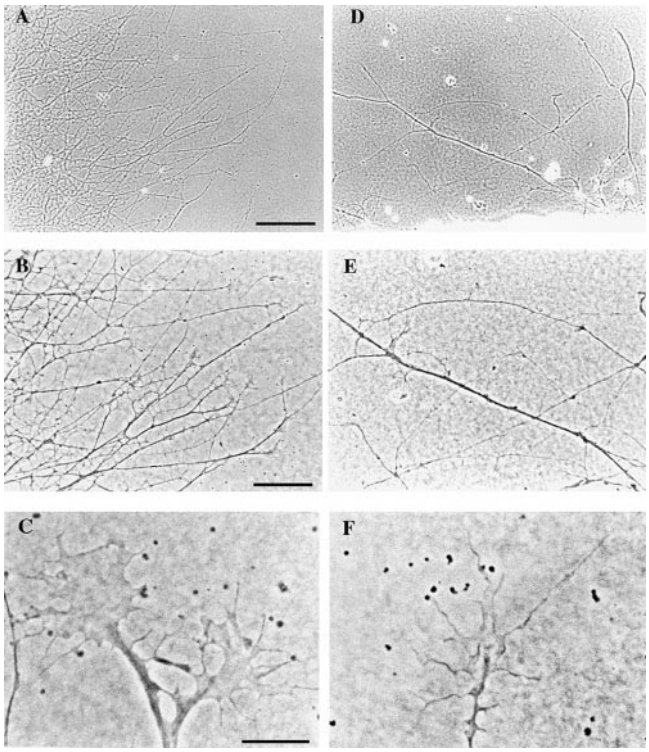

Figure 3. PTPμ promotes neurite outgrowth from chick retinal explants. Neural retina explants from E8 chick embryos were cultured for 2 d on N-cadherin–coated dishes (A–C) or 4 d on dishes coated with PTPμ (D–F). The fields shown in B and E are higher power images of the same fields shown in A and D, respectively. Long neurites grew out onto each substrate, but were more fasciculated on the PTPμ substrate (D and E) as compared with the N-cadherin substrate (A and B). Growth cones on N-cadherin were largely lamellipodial with several short filopodial processes (C), whereas growth cones on PTPμ were typically small and spiky in appearance with multiple long filopodial processes and a small lamellipodial region (F). Scale bars: (A) 100 μm, (B) 50 μm, (C) 10 μm.

substrate in comparison with the N-cadherin substrate may indicate that only a subset of RGC neurons are able to respond to PTPμ and induce neurite outgrowth. However, PTPμ was expressed at equal levels by all neurites growing on N-cadherin or PTPμ substrates (data not shown). The distinct morphology of neurites on a PTPμ substrate in comparison with N-cadherin suggests that PTPμ may use a unique signaling mechanism to promote neurite outgrowth.

To ensure that the neurite outgrowth activity in the purified PTPμ preparation was mediated by PTPμ, retinal explants were cultured on a PTPμ substrate and N-cadherin or L1 control substrates in the presence or absence of function-blocking antibodies against PTPμ, N-cadherin, or L1 (Fig. 4). Neurite outgrowth on a PTPμ substrate was completely blocked by the addition of antibodies against the extracellular portion of PTPμ (Fig. 4 B), but was unaffected by the addition of antibodies against N-cadherin (C) or L1 (D). These results demonstrate that PTPμ specifically promotes neurite outgrowth. N-cadherin antibodies caused a significant inhibition in neurite outgrowth on N-cadherin (Fig. 4 G). Antibodies against the extracellular domain of PTPμ had no effect on neurite outgrowth on a N-cadherin substrate (Fig. 4 F). Similarly, the L1 antibodies used for these experiments completely blocked neurite outgrowth on an L1 substrate (Fig. 4 K), but antibodies against PTPμ had no effect (J). Therefore, the antibodies used for these experiments were specific. Due to the longer time course to achieve neurite outgrowth on a PTPμ substrate (2–4 d), it was a concern that the retinal explants may secrete factors over time that could deposit on the dish surface and promote neurite outgrowth. For the culture of retinal explants on purified protein substrates, the dishes were first coated with substrate protein, and then the remaining binding sites on the dish were blocked by the addition of BSA before plating. A control experiment was done in which retinal explants were cultured on dishes coated with BSA alone. In this situation, no neurites were observed to extend from any explants over the course of 4 d (Fig. 4 H), suggesting that the neurite outgrowth–promoting activity was due to the purified protein used as the substrate and was not due to a soluble factor originating from the retina tissue.

To determine which cell types extended neurites on PTPμ, the cultures were fixed and processed for immunocytochemistry using antibodies against specific cell types of the retina. All of the long neurites growing on PTPμ were positively labeled with an antibody against L1 (8D9; Fig. 5, E and F), which is only expressed by the RGCs (Lemmon and McLoon, 1986), suggesting that the neurites on PTPμ were derived from RGC neurons. In addition, other cells were observed to migrate from the explants and were identified as bipolar neurons (based on reactivity to the 3G3 antibody; Fig. 5, A and B), or Müller glia (based on reactivity to the 5A7 antibody; Fig. 5, C and D) (Drazba and Lemmon, 1990). Other cells from E8 retinas,

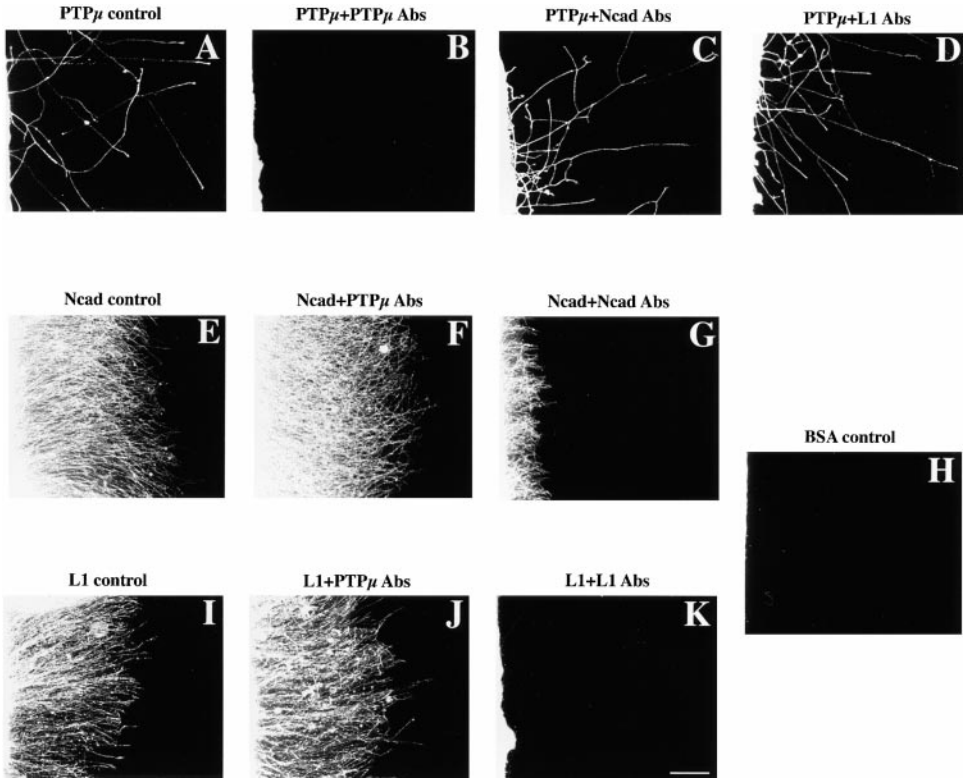

Figure 4. Neurite outgrowth on PTPμ is blocked by addition of antibodies against PTPμ. Neural retina explants from E8 chick embryos were cultured on a PTPμ (A–D), N-cadherin (E–G), or L1 (I–K) substrate in the presence of function-blocking antibodies against PTPμ (B, F, and J), N-cadherin (C and G), or L1 (D and K). The explants are on the left of each panel. The neurites in each dish were examined using dark-field optics. Antibodies against PTPμ inhibited neurite outgrowth on the PTPμ substrate (B), whereas they had no effect on neurite outgrowth on N-cadherin (F) or L1 substrates (J). Conversely, antibodies against N-cadherin inhibited neurite outgrowth on a N-cadherin substrate (G), but had no effect on neurite outgrowth on PTPμ (C). Similarly, antibodies against L1 inhibited neurite outgrowth on an L1 substrate (K), but had no effect on a PTPμ substrate (D). A dish coated with bovine serum albumin alone did not support neurite outgrowth (H), suggesting that, over time, the retinal explants did not secrete proteins that promote neurite outgrowth in this assay. Scale bar, 200 μm.

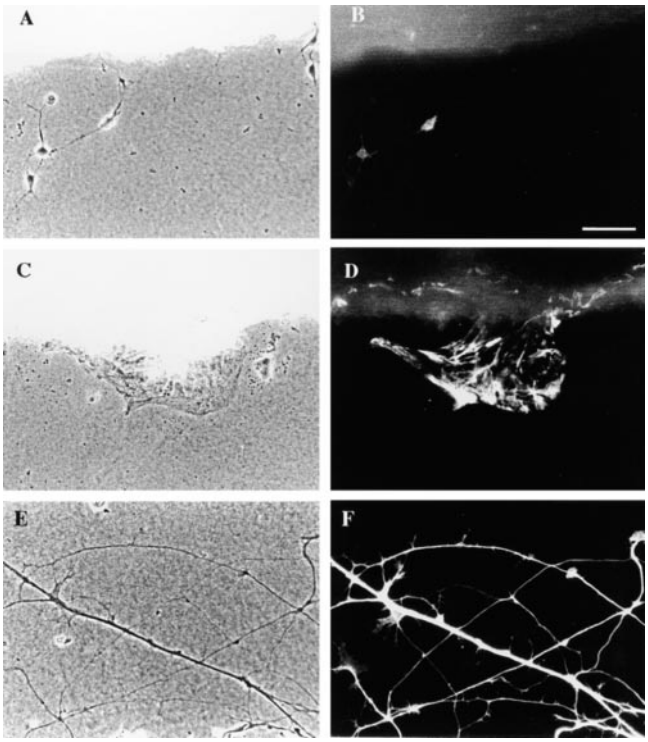

Figure 5. PTPμ promotes migration of several cell types from chick neural retina. Neural retina explants from E8 chick embryos were cultured on a PTPμ substrate for 4 d. The cells were fixed and immunolabeled with antibodies against a bipolar-specific antigen (A and B), a Müller glia-specific antigen (C and D), or L1 (E and F). Phase contrast (A, C, and E) and fluorescent images (B, D, and F) are shown. The long neurites that extended on the PTPμ substrate were from RGC neurons as shown by the labeling with an antibody against L1 (E and F), which is restricted to RGC axons in retina. Bipolar neurons (A and B) and Müller glia (C and D) were also observed to migrate onto PTPμ. Scale bar, 50 μm.

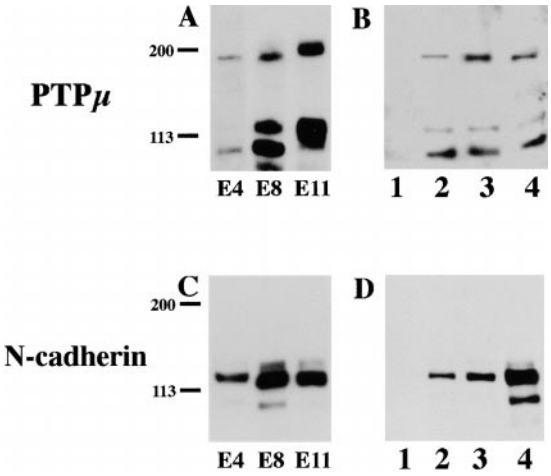

Figure 6. PTPμ interacts with N-cadherin in chick retina. Lysates were made from embryonic day 4, 8, and 11 neural retinas. 5 μg total protein per lane was separated by 6% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against (A) PTPμ or (C) N-cadherin. (B and D) Immunoprecipitates from lysates of E8 retina were immunoblotted. Mouse IgG (lane 1), PTPμ (lanes 2 and 3) and N-cadherin (lane 4) antibodies were used for immunoprecipitation. The immunoprecipitates were subjected to 6% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to (B) PTPμ or (D) N-cadherin.

such as photoreceptor cells, were not observed to migrate on PTPμ. The cells that grew out onto the PTPμ substrate all express PTPμ, suggesting that PTPμ may be acting homophilically to promote outgrowth.

PTPμ Interacts with N-Cadherin in Retina

PTPμ is abundant in chick retina, and its expression is developmentally regulated (Fig. 6 A). PTPμ expression is observed by E4 (the earliest time-point examined), with peak expression by E11. N-cadherin is also detected in retina at E4, and undergoes a similar increase in expression with development (Fig. 6 C) (Inuzuka et al., 1991). The molecular weight of PTPμ is altered with time, suggesting developmental regulation of glycosylation, proteolytic cleavage, and/or shedding. Similar modifications have been observed for a related RPTP, LAR (Streuli et al., 1992; Serra-Pages et al., 1994; Aicher et al., 1997).

To examine whether PTPμ associates with N-cadherin, the major cadherin in retina, immunoprecipitation experiments were done using E8 retina lysates. Similar results were obtained using E4 retinas (data not shown). Both the full-length (200 kD) and cleaved (100 kD) forms of PTPμ were immunoprecipitated by antibodies to PTPμ (Fig. 6 B, lanes 2 and 3), but not by mouse IgG (Fig. 6 B, lane 1). When immunoprecipitates of PTPμ were probed on immunoblots with antipancadherin antibodies, an association with N-cadherin was detected (Fig. 6 D, lanes 2 and 3). In the reciprocal experiment, N-cadherin was immunoprecipitated by antibodies to N-cadherin (Fig. 6 D, lane 4), but was not detected when mouse IgG was used for immunoprecipitation (lane 1). N-cadherin immunoprecipitates contained the 200-kD full-length form of PTPμ (Fig. 6 B, lane 4), and the 100-kD fragment of PTPμ that was also present in the PTPμ immunoprecipitates (lanes 2 and 3).

The results from the immunoprecipitation experiments indicated that PTPμ associates with N-cadherin. Since PTPμ is expressed at high levels within RGC neurons, it is likely that an association with N-cadherin occurs within these cells. To verify that PTPμ interacts with cadherins in RGC neurons, an antibody cross-linking experiment was performed using neurites from E8 chick retinal explants growing on the control substrate laminin. In Fig. 7, A–B and E–F, the cells were fixed and processed for immunocytochemistry using the 494 polyclonal antibody against PTPμ (A and E) and a monoclonal antibody against either N-cadherin (B) or NCAM (F). In the absence of PTPμ cross-linking, N-cadherin, NCAM, and PTPμ were present in a continuous fashion along the length of the axons and growth cones. For the cross-linking experiments, the explants were cultured for 24 h to allow significant RGC neurite outgrowth, and the live cultures were incubated with a polyclonal antibody against the extracellular domain of PTPμ (494) to permit clustering of PTPμ protein on the cell surface (patching; Fig. 7, C and G). The cells were then fixed and processed for immunocytochemistry with antibodies to N-cadherin (Fig. 7 D) or NCAM (H). The PTPμ protein was cross-linked into patches along the

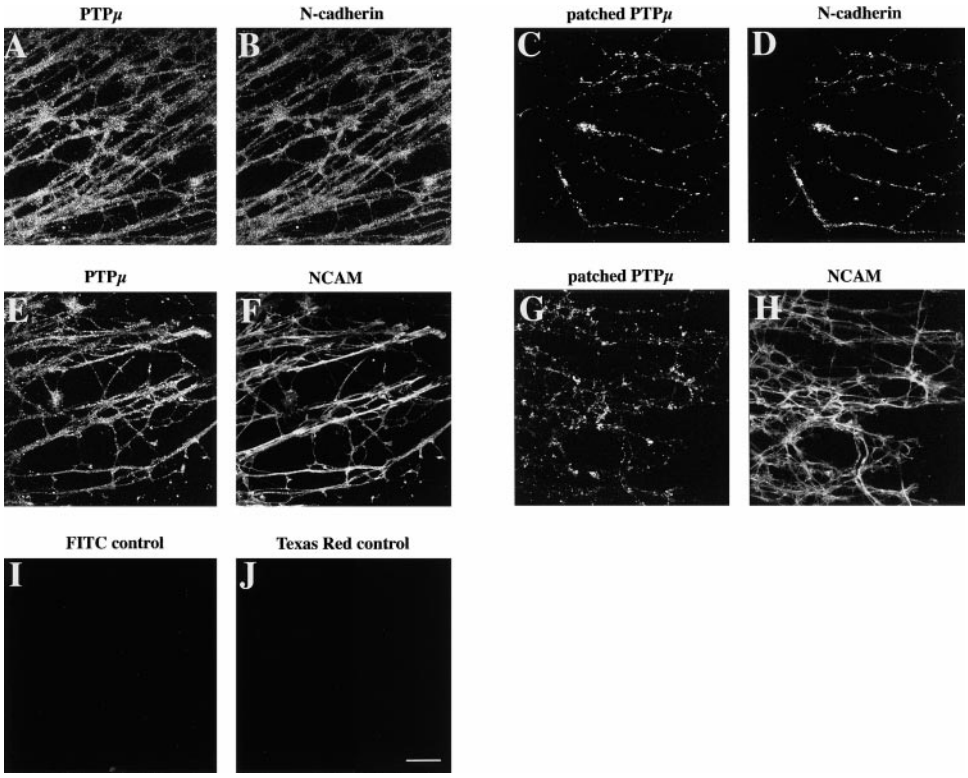

Figure 7. PTPμ colocalizes with N-cadherin in retinal neurites. Neural retina explants from E8 chick embryos were cultured overnight on laminin. The cells were fixed and double labeled with a polyclonal antibody against PTPμ (A and E) and a monoclonal antibody against either N-cadherin (B) or NCAM (F). These results show that PTPμ, N-cadherin, and NCAM are expressed in a continuous fashion along neurites and into growth cones. Live cells were incubated with a polyclonal antibody against PTPμ to cluster the PTPμ protein on the surface of the neurites into "patches" (C and G), and then the cells were fixed and double labeled with a monoclonal antibody against N-cadherin (D) or NCAM (H). N-cadherin (D), but not NCAM (H), colocalized to the PTPμ patch sites, suggesting an interaction with PTPμ within the neurites. As a control for nonspecific fluorescence from the secondary antibodies, fixed retina cultures were processed for immunocytochemistry as above except that the primary antibodies were omitted (I and J). The image shown in I is a control for images shown in A, C, E, and G, whereas the image shown in J is a control for images shown in B, D, F and H. The control images (I and J) were collected using identical settings on the confocal microscope as those used for the corresponding experimental images (A–H). Scale bar, 20 μm.

neurites (Fig. 7, C and G). Importantly, N-cadherin protein also became concentrated into the PTPμ patch sites (Fig. 7 D). Extensive colocalization was observed between PTPμ and N-cadherin, suggesting a stoichiometric association between these proteins in neurites. In contrast, another abundantly expressed cell adhesion molecule, NCAM, did not become concentrated into PTPμ patch sites (Fig. 7 H). The results of these experiments show that PTPμ and N-cadherin associate in the RGC neurites, and provide a basis for examination of the function of that association in neurite outgrowth.

PTPμ Is Required for N-Cadherin–dependent Neurite Outgrowth

Based on previous studies, we hypothesized that PTPμ maintains a protein in the N-cadherin/catenin complex in a dephosphorylated state that may be important for N-cadherin–mediated adhesion and/or neurite outgrowth. To examine the role of PTPμ in N-cadherin–mediated adhesion events, a retrovirus encoding the antisense version of the PTPμ cDNA sequence was used to downregulate PTPμ expression in dissociated retinal cells. PTPμ is normally synthesized as a full length (200 kD) precursor that can be expressed at the cell surface or cleaved into a 105 kD form (predominately extracellular) or 100 kD form (the intracellular domain, transmembrane region, and a short stretch of the extracellular domain; Brady-Kalnay and Tonks, 1994a; Gebbink et al., 1995). Immunoblot analysis and densitometric measurement of the gel bands that were immunoreactive with an antibody against PTPμ demonstrated that the full-length protein (200-kD band) was reduced by 99% in cells infected with PTPμ antisense virus when compared with cells infected with control virus (Fig. 8 A, lanes 1 and 3). In addition, the 100-kD band was reduced by 77%, whereas a 95-kD immunoreactive band was unchanged (Fig. 8 A, lane 3). These results suggest that PTPμ antisense expression inhibits the new synthesis of PTPμ. Therefore, the full-length protein made before infection has likely been cleaved over time to form the two smaller fragments that may not turn over rapidly. The retrovirus used for these experiments is negatively regulated by tetracycline. When tetracycline was present in the medium, PTPμ antisense virus had no effect on PTPμ expression level (Fig. 8 A, lane 4). In contrast, no change in N-cadherin expression was detected in cells infected with PTPμ antisense virus when compared with cells infected with control virus (Fig. 8 B). The blot for PTPμ expression was stripped and reprobed with antibodies to NCAM to verify equal protein loading per lane (Fig. 8 C). These results demonstrate that the PTPμ antisense retrovirus was able to infect the retina cells, resulting in a significant downregulation of PTPμ expression.

To determine whether PTPμ function is required for N-cadherin–mediated neurite outgrowth, E4 retinas were infected with PTPμ antisense or control retrovirus. Explants from the retinas were then cultured on N-cadherin

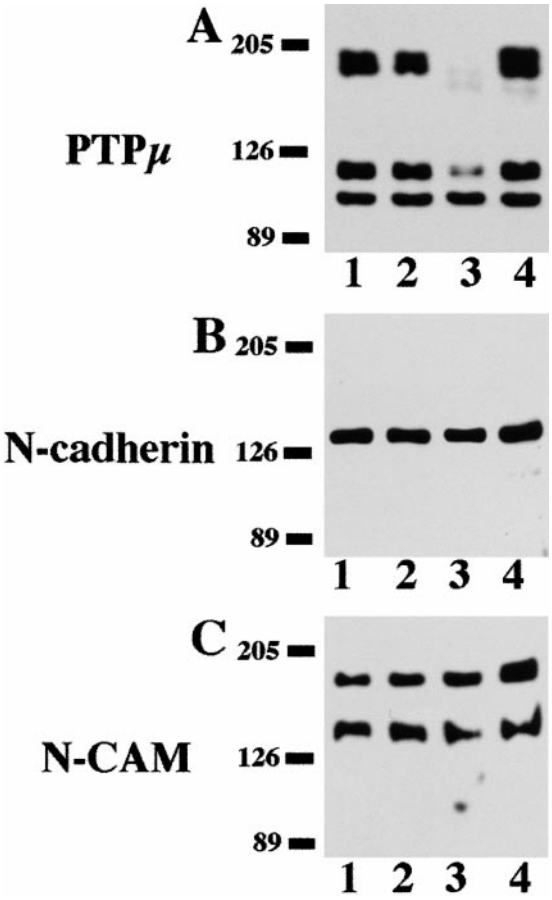

Figure 8. PTPμ expression is reduced in retinal cells infected with antisense PTPμ retrovirus. Dissociated chick retinal cells infected with control virus (lanes 1 and 2) or PTPμ antisense virus (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 3 μg/ml tetracycline were harvested 72 h after infection. 7.5 μg of each lysate was separated by 6% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to (A) PTPμ, (B) N-cadherin, or (C) NCAM. (A) A substantial reduction in PTPμ expression was detected in lysates from PTPμ antisense–infected cells in the absence of tetracycline (lane 3) in comparison with lysates from control virus–infected cells (lane 1). Densitometric measurement of the PTPμ bands showed a 99% reduction of the 200-kD band and a 77% reduction of the 100-kD band, whereas a 95-kD band was unchanged. No difference in PTPμ expression was observed when cells infected with PTPμ antisense were cultured in the presence of tetracycline (lane 4). (B) In contrast, no difference in N-cadherin expression was detected in lysates from control virus (lanes 1 and 2) or PTPμ antisense virus (lanes 3 and 4) infected cells. (C) The blot probed for PTPμ expression was stripped and reprobed for a nonspecific protein, NCAM, to verify equal protein loading per lane.

or control substrates and neurite outgrowth was examined at 24 and 48 h after plating. E4 retinas were used for these experiments because a large percentage of the cells are still mitotic at this age, which is a requirement for retroviral-mediated gene transfer. Explants infected with PTPμ antisense virus displayed a dramatic and significant decrease in neurite outgrowth on N-cadherin in comparison with sister explants infected with control virus (Figs. 9, C–D, and 10, A–B; Table I). A range of effects was detected, including no neurite outgrowth, some short neurites, and in a few cases a small number of long neurites were observed. After infection with antisense virus, there is an ~50% reduction in PTPμ expression in the neurites when they are immunolabeled with antibodies to PTPμ (data not shown). Therefore, there is a substantial reduction in the PTPμ phosphatase overall, but some residual expression of PTPμ in all of the neurites in the explant population. The photograph shown (Fig. 9, C–D) is representative of the median level of neurite outgrowth. Quantitation of all of the results indicated that neurite lengths were reduced by 53% and overall neurite density was reduced by 74% in cultures expressing PTPμ antisense in comparison with cells expressing control virus. In contrast, PTPμ antisense had no effect on either length or density of neurites growing on the control substrates laminin (Figs. 9, A–B, and 10, A–B), or L1 (Figs. 9, E–F, and 10, A–B; Table I). Since the length and density of outgrowth on laminin and L1 in the presence of PTPμ antisense virus was similar to that observed previously (Lemmon et al., 1992), it seems unlikely that the PTPμ antisense virus altered RGC differentiation. In addition, no difference in the level of axon fasciculation was observed in cultures infected with control versus PTPμ antisense virus on any substrate examined. More importantly, the ability of neurons to extend neurites on other substrates suggests that the PTPμ antisense virus was not toxic for the neurons themselves and did not have nonspecific effects on neurite outgrowth in general. These results suggest that PTPμ is specifically involved in regulating N-cadherin–mediated neurite outgrowth.

The retroviral expression system used for these experiments is repressed in the presence of tetracycline (Paulus et al., 1996). Retinal explants plated on N-cadherin in the presence of both tetracycline and PTPμ antisense virus showed no reduction in either neurite length or density (Fig. 10, A–B; Table I) when compared with explants infected with control virus. These results demonstrate that expression of PTPμ antisense is regulated by the presence of tetracycline. In addition, the observed decrease in neurite outgrowth on N-cadherin requires the expression of antisense PTPμ.

PTPμ is capable of acting as both an adhesion molecule and an enzyme; therefore, it was important to determine which of these functions was necessary for the regulation of N-cadherin–mediated neurite outgrowth. To address this issue, we generated a PTPμ mutant that contained a single amino acid change (cysteine to serine) in the catalytic domain of the phosphatase. This c → s mutant, which encoded the full-length PTPμ protein tagged at the COOH terminus with GFP, preserved the adhesive function of the extracellular segment but rendered the phosphatase catalytically inactive. E4 retinas were infected with a retrovirus encoding the c → s mutant form of PTPμ, and cultured on an N-cadherin substrate. Explants infected with the c → s mutant virus displayed a dramatic and significant decrease (~50%) in neurite outgrowth on N-cadherin in comparison with sister explants infected with control virus (Fig. 10, C–D; Table I). These results were similar to those obtained in cultures infected with PTPμ antisense virus (Fig. 10, A–B; Table I). Overexpression of full-length PTPμ coupled to GFP did not alter neurite outgrowth on N-cadherin (Fig. 10, C–D; Table I). These results indicate that PTPμ tyrosine phosphatase

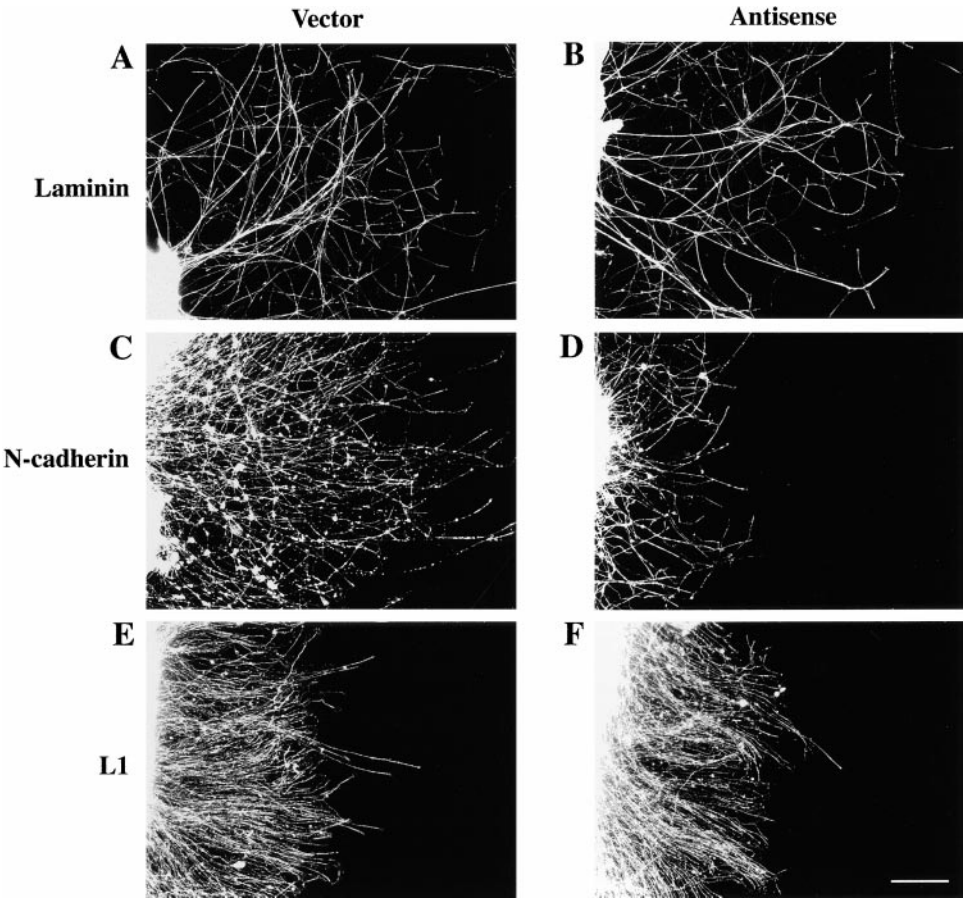

Figure 9. PTPμ is required for N-cadherin–dependent neurite outgrowth. E4 chick retina explants were infected with control (A, C, and E) or PTPμ antisense (B, D, and F) virus and cultured on laminin (A and B), N-cadherin (C and D), or L1 (E and F) substrates for 48 h. The neurites in each dish were examined using dark-field optics. No difference in neurite length or density was observed in cultures infected with vector or PTPμ antisense virus when cultured on laminin (A and B) or L1 (E and F) substrates. In contrast, PTPμ antisense resulted in a dramatic reduction of both neurite length and density in cultures on a N-cadherin substrate (C and D). Scale bar, 400 μm for images shown in A and B. Scale bar, 200 μm for images shown in C–F.

activity is a key regulatory component for proper N-cadherin function.

Discussion

To gain an understanding of the function of PTPμ in the developing nervous system, we examined the ability of embryonic chick retinal neurons to extend neurites on a PTPμ substrate. PTPμ promoted neurite outgrowth from RGC neurons, and the migration of bipolar neurons and Müller glia from E8 retinal explants. Neurite outgrowth on a PTPμ substrate was blocked by the addition of antibodies against PTPμ, indicating that the neurite outgrowth activity in the purified PTPμ preparation was specific to

Table I. Statistical Analysis of Retroviral-mediated Perturbation of PTPμ on Neurite Outgrowth

Substrate	Virus type	Length (μm)	*P	Density (pixels)	*P
		± *SEM*		± *SEM*	
Laminin (n = 20)	Vector	2118.7 ± 33.6		460727.6 ± 38294.4	
	Antisense	2120.4 ± 43.0	0.9764	475456.6 ± 47267.0	0.8066
L1 (n = 21)	Vector	718.3 ± 28.9		588344.8 ± 97990.8	
	Antisense	677.5 ± 33.8	0.2314	635546.3 ± 105779.5	0.6659
N-cadherin (n = 37)	Vector	1244.2 ± 31.1		345088.8 ± 47357.5	
	Antisense	583.2 ± 38.5	<0.0001	88339.5 ± 18692.3	<0.0001
N-cadherin + Tet (n =11)	Vector	1270.5 ± 66.6		328013.9 ± 75864.6	
	Antisense	1210.1 ± 85.2	0.4828	330952.2 ± 86113.2	0.9785
N-cadherin (n = 20)	Vector	1058.0 ± 49.4		313659.3 ± 61925.4	
	PTPμGFP	1039.4 ± 48.8	0.7892	352796.6 ± 54739.4	0.6385
N-cadherin (n = 18)	Vector	1445.1 ± 41.8		432560.8 ± 52316.9	
	C → S mutant	833.1 ± 62.4	<0.0001	179565.2 ± 44197.4	0.0008

n, number of retinal explants tested per virus type on that substrate; **P*, values from Fisher's PLSD test, 99% confidence interval.

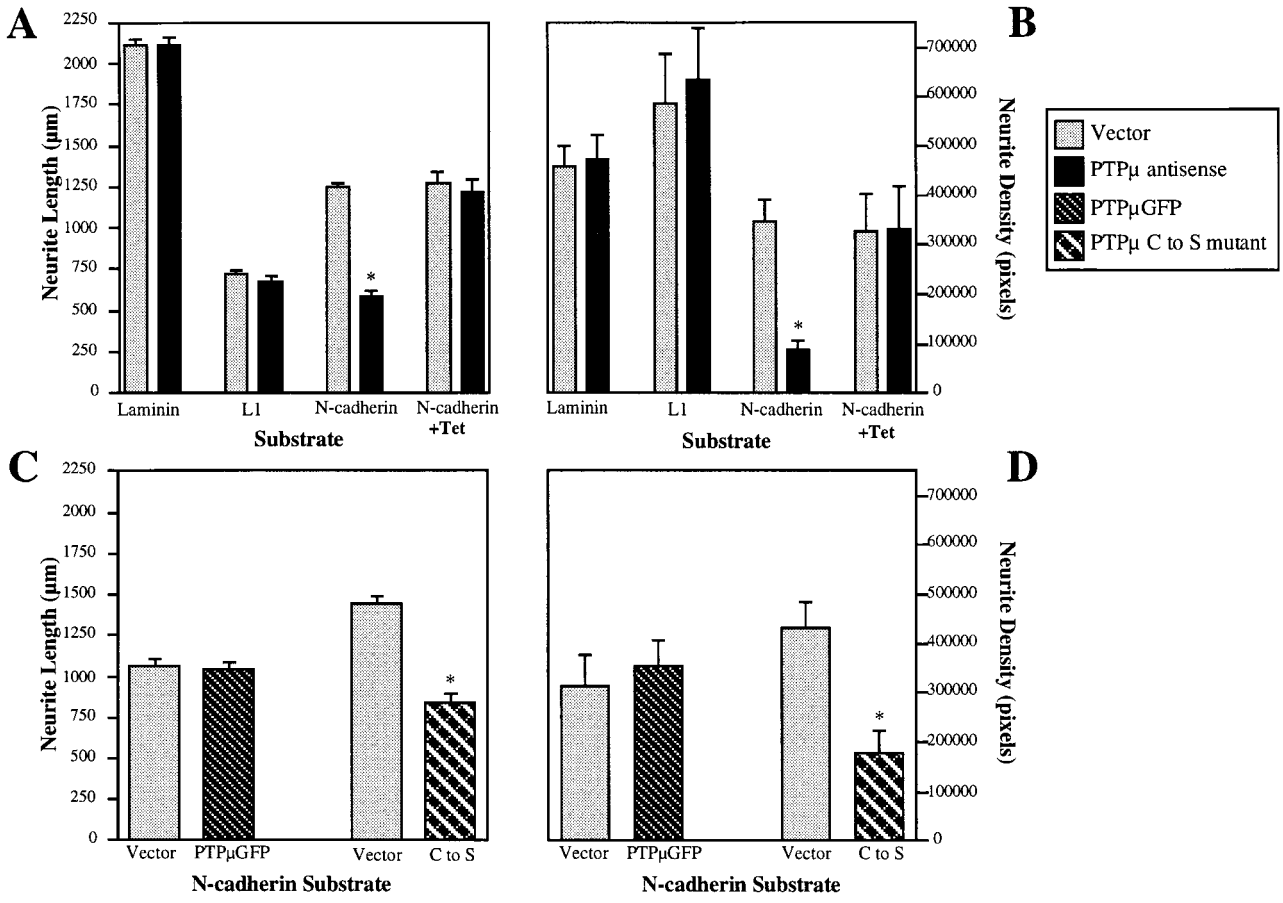

Figure 10. Quantitation of the effect of PTPμ perturbation on neurite outgrowth. E4 chick retina explants were infected with control virus (gray bars) or PTPμ antisense virus (black bars) and cultured on laminin, N-cadherin, or L1 substrates for 48 h. PTPμ antisense virus caused a significant reduction in neurite length (A) and density (B) on N-cadherin, but not on laminin or L1 substrates. The retrovirus used is negatively regulated by tetracycline (tet-off system). Retinal explants plated on N-cadherin in the presence of both tetracycline and PTPμ antisense virus showed no reduction in either neurite length (A) or density (B) when compared with explants infected with control virus. For statistical analysis, see Table I. E4 chick retinas were infected with control virus (gray bars) or test virus encoding the c → s (C to S) mutant of PTPμ (cross-hatched bars) or PTPμGFP sense control (striped bars), and cultured on N-cadherin for 48 h. The c → s mutant caused a significant reduction in neurite length (C) and density (D), whereas the sense control had no effect on either length or density. For statistical analysis, see Table I.

the PTPμ protein. The morphology of neurites growing on PTPμ was unique from that observed on other purified CAMs, suggesting PTPμ may use a distinct signaling mechanism to promote neurite outgrowth. Within the retina, the expression of PTPμ is developmentally regulated and increases over time in a pattern similar to N-cadherin expression (Matsunaga et al., 1988; Inuzuka et al., 1991). Previous studies have demonstrated that cadherins and PTPμ associate with one another (Brady-Kalnay et al., 1995, 1998; Hiscox and Jiang, 1998). In this study, we demonstrated that PTPμ and N-cadherin form a complex in retinal tissue. An association between PTPμ and N-cadherin was also demonstrated in RGC neurites, suggesting that PTPμ may be involved in the regulation of N-cadherin–mediated neurite outgrowth. The downregulation of PTPμ expression through antisense techniques resulted in a decreased ability of RGC neurites to extend on a N-cadherin substrate, but did not affect neurite outgrowth on laminin or L1 substrates. Overexpression of a catalytically inactive form of PTPμ also inhibited N-cadherin–mediated neurite outgrowth, thus providing further evidence that a component of the N-cadherin/catenin complex may be a substrate of PTPμ. Together, these results provide evidence that PTPμ is capable of promoting neurite outgrowth individually, and specifically regulating neurite outgrowth mediated by N-cadherin.

Axonal pathfinding, fasciculation, target recognition, and synapse formation are all processes that require contact-mediated recognition of cell surface cues. The diversity of the CAMs and other molecules involved in axonal pathfinding reflects the staggering array of decisions an individual axon must make along the way to its target. Many of the guidance molecules are members of the immunoglobulin superfamily. These include CAMs like L1 and NCAM, tyrosine kinases such as Eph family members and FGF receptors and even some RPTPs; for example, DLAR, DPTP69D, and now PTPμ. Presumably, these molecules mediate specific recognition events at different

points during axonal outgrowth and pathfinding. CAMs are not solely involved in adhesion of neurons to one another; they also participate in signal transduction. The interaction of a growth cone with a particular CAM can lead to rapid and specific changes in growth cone morphology (Burden-Gulley et al., 1995). This implies the adhesion molecules are sending signals that result in a transient change in the underlying cytoskeleton (Burden-Gulley and Lemmon, 1996) that guide a neuron toward its target (Lin and Forscher, 1993; Bentley and O'Connor, 1994).

RPTPs Are Involved in Axonal Guidance

Previous inhibitor studies suggested that tyrosine phosphatases are involved in the control of neurite outgrowth in general and on CAM substrates (Bixby and Jhabvala, 1992; Beggs et al., 1994; Ignelzi et al., 1994). Recent studies suggest that regulation of tyrosine phosphorylation by RPTPs affects axonal growth possibly by "steering" growth cones along the appropriate pathway (Desai et al., 1997). In *Drosophila,* two CAM-like RPTPs are expressed in the central nervous system, and knockout experiments have demonstrated that they play critical roles in development. Mutant embryos for the *Drosophila* RPTPs, DLAR and DPTP69D, display an inability of specific motorneurons to recognize guidance cues that allow them to innervate appropriate target muscles (Desai et al., 1996; Krueger et al., 1996). In addition, a LAR homologue in leech was shown to accumulate in a subset of axonal growth cones and play a guidance role during outgrowth of these axonal processes (Gershon et al., 1998). Together with the present study, these data provide the first evidence that RPTPs could be directly involved in axonal pathfinding and suggest that tyrosine phosphorylation is a key regulator of axonal guidance and choice point recognition.

In the present study, we demonstrated that PTPμ plays a role in neurite outgrowth at physiological levels of protein expression. This data is important because previous studies on the ability of PTPμ to mediate aggregation were performed when PTPμ was massively overexpressed (Brady-Kalnay et al., 1993). We have now demonstrated that PTPμ promotes neurite outgrowth from RGC neurons, presumably through a homophilic binding mechanism. PTPμ may have several important roles in nervous system development. First, it may act as a cell–cell adhesion molecule necessary for maintenance of nervous system integrity. This could occur through homophilic binding of PTPμ on the surfaces of two apposing axons to promote axon fasciculation, a process required for nerve formation. A similar role has been suggested for other CAMs such as L1 (Lemmon et al., 1989; Tang et al., 1992) and NCAM (Rutishauser et al., 1978). Second, PTPμ may act as a permissive molecule for axonal growth. The expression of PTPμ by axons, astrocytes (our unpublished data) and other nonneuronal cells makes this role a likely possibility. A contact attraction role for PTPμ is a third possibility, such that PTPμ actively guides axons during pathfinding. For example, PTPμ may be expressed at specific choice points where axons must choose the appropriate pathway. RGC growth cones encounter several choice points during outgrowth to their target, the optic tectum (Tessier-Lavigne, 1995). The mechanisms regulating this stereotypical innervation pattern are only partly understood, but involve tyrosine phosphorylation (Cox et al., 1990; Drescher et al., 1997). A fourth possible function of PTPμ is as a sensor molecule. For example, changes in the adhesive state of the extracellular environment may be transmitted through PTPμ via regulation of its catalytic domain. PTPμ may then directly regulate the phosphorylation state of a number of cytosolic proteins, including components of the cadherin/catenin complex. This idea is supported by the inhibition of N-cadherin–mediated neurite outgrowth when RGC neurons overexpress a catalytically inactive form of PTPμ.

Cadherins Are Regulated by Tyrosine Phosphorylation

A likely target of the PTPμ enzyme is a component of the N-cadherin complex, which was previously postulated to be a substrate of PTPμ (Brady-Kalnay et al., 1995, 1998). The association of cadherins and catenins with receptor and nontransmembrane PTPs has now been observed by many groups (Brady-Kalnay et al., 1995; Balsamo et al., 1996, 1998; Fuchs et al., 1996; Kypta et al., 1996; Aicher et al., 1997; Cheng et al., 1997; Brady-Kalnay et al., 1998; Hiscox and Jiang, 1998). The juxtamembrane domains of PTPκ (Fuchs et al., 1996) and PCP2 (Cheng et al., 1997) interact with β catenin. A LAR-like RPTP associates with the cadherin/catenin complex in PC12 cells (Kypta et al., 1996) and the intracellular domain of LAR was shown to bind directly to β catenin and plakoglobin in vitro (Aicher et al., 1997). The PTP1B cytoplasmic phosphatase was shown to interact with the N-cadherin/catenin complex and dephosphorylate β catenin (Balsamo et al., 1996), a process required for N-cadherin–mediated adhesion and actin linkage (Balsamo et al., 1998). Therefore, it is likely that regulation of the cadherin/catenin complex by PTPs will be an important mechanism of control in many cell types, including neurons.

Tyrosine phosphorylation of the cadherin/catenin complex correlates with suppression of cadherin-mediated adhesion (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993), adherens junction disassembly/loss of cytoskeletal association (Warren and Nelson, 1987; Volberg et al., 1991, 1992), invasion and malignant progression (Kemler, 1993; Birchmeier, 1995). Tyrosine phosphorylation of the cadherin/catenin complex can be catalyzed by pp60src, EGF receptor, c-erbB2, or hepatocyte growth factor receptor (Tsukita et al., 1991; Hoschuetzky et al., 1994; Ochiai et al., 1994; Shibamoto et al., 1994). These data indicate that one of the mechanisms the cell uses to regulate the function of the cadherin/catenin complex is tyrosine phosphorylation. In the present study, PTPμ associated with N-cadherin in lysates from retina as demonstrated by immunoprecipitation techniques. A similar association was demonstrated in RGC neurites through antibody cross-linking and immunocytochemistry techniques. In addition, the ability of neurites to migrate on N-cadherin was significantly impaired when PTPμ expression was downregulated. These results provide evidence that N-cadherin–mediated neurite outgrowth requires functional PTPμ. The inhibition of N-cadherin–mediated neurite outgrowth due to overexpression of the catalytically inactive form of PTPμ further supports the idea that cadherins or their associated proteins need to be dephosphorylated to function in adhesion (Brady-Kalnay et al., 1995, 1998).

Therefore, PTPμ tyrosine phosphatase activity is a key regulatory component of the N-cadherin/catenin complex. In contrast, PTPμ downregulation did not alter neurite outgrowth on L1 or laminin control substrates. These results suggest that the effects of PTPμ downregulation were specific to N-cadherin–mediated neurite outgrowth and not due to general alterations in cellular phosphotyrosine that could nonspecifically affect neurite outgrowth.

Perspectives on the Developmental Role of PTPμ in the Retinotectal System

Our data demonstrate that PTPμ can promote neurite outgrowth, which is consistent with a role for PTPμ in neuronal pathfinding. This promotion of neurite outgrowth could be mimicking the ability of certain neurons to respond to signaling events initiated by PTPμ. The inability of PTPμ to promote neurite outgrowth from retinas earlier than E6 suggests that a threshold level of PTPμ expression on axons may be required for PTPμ to independently promote neurite outgrowth through a homophilic mechanism. At earlier ages, PTPμ may play other specific roles; for example, regulation of N-cadherin–dependent adhesion that is required for morphogenetic movements or axonal pathfinding events. N-cadherin is one of the key molecules involved in many aspects of retinal function from histogenesis and lamination to neurite outgrowth and synapse formation (Matsunaga et al., 1988; Redies and Takeichi, 1993; Fannon and Colman, 1996). PTPμ may regulate N-cadherin function by modulating signals that allow neurons to respond to N-cadherin–mediated adhesion.

A number of individuals provided assistance with this study, and their efforts were greatly appreciated. These include Dr. Leif Stordal, who generated the retroviral plasmids used for the antisense experiments, and Tracy Mourton and Ferdouse Sartawi, who provided expert technical assistance. The many contributions of Dr. Vance Lemmon throughout the course of this study are greatly appreciated. In addition, we thank Drs. Lloyd Culp, Lynn Landmesser, Gary Landreth, Vance Lemmon, and Robert Miller for helpful comments on the manuscript.

This work was supported by Research Oncology Training Grant CA-59366-04 to S. Burden-Gulley, and a grant from the American Cancer Society, Ohio Division, Cuyahoga County Unit, and National Institutes of Health grant 1RO1-EY12251 to S. Brady-Kalnay. Additional support was provided by the Visual Sciences Research Center Core Grant from the National Eye Institute (PO-EY11373).

Received for publication 15 June 1998 and in revised form 3 February 1999.

References

Aberle, H., H. Schwartz, and R. Kemler. 1996. Cadherin–catenin complex: protein interactions and their implications for cadherin function. *J. Cell. Biochem*. 61:514–523.

Aicher, B., M.M. Lerch, T. Muller, J. Schilling, and A. Ulrich. 1997. Cellular redistribution of protein tyrosine phosphatases LAR and PTPσ by inducible proteolytic processing. *J. Cell Biol.* 138:681–696.

Balsamo, J., T.C. Leung, H. Ernst, M.K.B. Zanin, S. Hoffman, and J. Lilien. 1996. Regulated binding of a PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of β catenin. *J. Cell Biol.* 134:801–813.

Balsamo, J., C. Arregui, T.C. Leung, and J. Lilien. 1998. The nonreceptor protein tyrosine phosphatase PTP1B binds to the cytoplasmic domain of N-cadherin and regulates the cadherin–actin linkage. *J. Cell Biol.* 143:523–532.

Barford, D., A.J. Flint, and N.K. Tonks. 1994. Crystal structure of human protein tyrosine phosphatase 1B. *Science*. 263:1397–1404.

Beggs, H.E., P. Soriano, and P.F. Maness. 1994. N-CAM-dependent neurite outgrowth is inhibited in neurons from fyn-minus mice. *J. Cell Biol.* 127: 825–833.

Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β catenin complex in cells transformed with a temperature-sensitive v-src gene. *J. Cell Biol.* 120:757–766.

Bentley, D., and T.P. O'Connor. 1994. Cytoskeletal events in growth cone steering. *Curr. Opin. Neurobiol*. 4:43–48.

Birchmeier, W. 1995. E-cadherin as a tumor (invasion) suppressor gene. *Bioessays*. 17:97–99.

Bixby, J.L., and P. Jhabvala. 1992. Inhibition of tyrosine phosphorylation potentiates substrate-induced neurite growth. *J. Neurobiol*. 23:468–480.

Bixby, J.L., and R. Zhang. 1990. Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. *J. Cell Biol*. 110:1253–1260.

Brady-Kalnay, S., A.J. Flint, and N.K. Tonks. 1993. Homophilic binding of PTPμ, a receptor-type protein tyrosine phosphatase, can mediate cell–cell aggregation. *J. Cell Biol.* 122:961–972.

Brady-Kalnay, S., and N.K. Tonks. 1994a. Identification of the homophilic binding site of the receptor protein tyrosine phosphatase PTPμ. *J. Biol. Chem*. 269:28472–28477.

Brady-Kalnay, S.M., and N.K. Tonks. 1994b. Protein tyrosine phosphatases: from structure to function. *Trends Cell Biol.* 4:73–76.

Brady-Kalnay, S.M., D.L. Rimm, and N.K. Tonks. 1995. The receptor protein tyrosine phosphatase PTPμ associates with cadherins and catenins *in vivo*. *J. Cell Biol.* 130:977–986.

Brady-Kalnay, S.M., and N.K. Tonks. 1995. Protein tyrosine phosphatases as adhesion receptors. *Curr. Opin. Cell Biol.* 7:650–657.

Brady-Kalnay, S.M., T. Mourton, J.P. Nixon, G.E. Pietz, M. Kinch, H. Chen, R. Brackenbury, D.L. Rimm, R.L. Del Vecchio, and N.K. Tonks. 1998. Dynamic interaction of PTPμ with multiple cadherins in vivo. *J. Cell Biol.* 141: 287–296.

Brady-Kalnay, S. 1998. Ig superfamily phosphatases. *In* Ig Superfamily Molecules in the Nervous System. Vol. 6. P. Sonderegger, editor. Harwood Academic Publishers, Amsterdam, Netherlands. 133–159.

Brittis, P.A., V. Lemmon, U. Rutishauser, and J. Silver. 1995. Unique changes of ganglion cell growth cone behavior following cell adhesion molecule perturbations: a time-lapse study of the living retina. *Mol. Cell. Neurosci*. 6:433–449.

Brittis, P.A., and J. Silver. 1995. Multiple factors govern intraretinal axon guidance: a time-lapse study. *Mol. Cell. Neurosci*. 6:413–432.

Brummendorf, T., and F.G. Rathjen. 1993. Axonal glycoproteins with immunoglobulin- and fibronectin type III–related domains in vertebrates: structural features, binding activities, and signal transduction. *J. Neurochem*. 61:1207–1219.

Brummendorf, T., and F. Rathjen. 1998. Molecular interactions involving immunoglobulin superfamily adhesion proteins. *In* Ig Superfamily Molecules in the Nervous System. Vol. 6. P. Sonderegger, editor. Harwood Academic Publishers, Amsterdam, Netherlands. 23–56.

Burden-Gulley, S.M., and V. Lemmon. 1995. Ig superfamily adhesion molecules in the vertebrate nervous system; binding partners and signal transduction during axon growth. *Semin. Dev. Biol.* 6:79–87.

Burden-Gulley, S.M., and V. Lemmon. 1996. L1, N-cadherin and laminin induce distinct distribution patterns of cytoskeletal elements in growth cones. *Cell Motil. Cytoskelet.* 35:1–23.

Burden-Gulley, S.M., H.R. Payne, and V. Lemmon. 1995. Growth cones are actively influenced by substrate-bound adhesion molecules. *J. Neurosci.* 15: 4370–4381.

Cheng, J., K. Wu, M. Armanini, N. O'Rourke, D. Dowbenko, and L.A. Lasky. 1997. A novel protein-tyrosine phosphatase related to the homotypically adhering κ and μ receptors. *J. Biol. Chem*. 272:7264–7277.

Cox, E.C., B. Muller, and F. Bonhoeffer. 1990. Axonal guidance in the chick visual system: posterior tectal membranes induce collapse of growth cones from the temporal retina. *Neuron*. 4:31–37.

Culp, L.A., K.L. O'Connor, and R. Lechner. 1997. Extracellular matrix adhesion: biological, molecular and pathogenic mechanisms. *In* Principles of Medical Biology. Vol. 7B. JAI Press Inc., Greenwich, CT. 573–607.

Desai, C., J.G. Gindhart, L.S.B. Goldstein, and K. Zinn. 1996. Receptor tyrosine phosphatases are required for motor axon guidance in the *Drosophila* embryo. *Cell*. 84:599–609.

Desai, C.J., Q. Sun, and K. Zinn. 1997. Tyrosine phosphorylation and axon guidance: of mice and flies. *Curr. Opin. Neurobiol.* 7:70–74.

Drazba, J., and V. Lemmon. 1990. The role of cell adhesion molecules in neurite outgrowth on Müller cells. *Dev. Biol.* 138:82–93.

Drescher, U., F. Bonhoeffer, and B.K. Muller. 1997. The Eph family in retinal axon guidance. *Curr. Opin. Neurobiol.* 7:75–80.

Fannon, A.M., and D.R. Colman. 1996. A model for central synaptic junctional complex formation based on the differential adhesive specificities of the cadherins. *Neuron*. 17:423–434.

Fuchs, M., T. Muller, M.M. Lerch, and A. Ulrich. 1996. Association of human protein-tyrosine phosphatase κ with members of the armadillo family. *J. Biol. Chem*. 271:16712–16719.

Gebbink, M., I. van Etten, G. Hateboer, R. Suijkerbuijk, R. Beijersbergen, A. van Kessel, and W. Moolenaar. 1991. Cloning, expression and chromosomal localization of a new putative receptor-like protein tyrosine phosphatase. *FEBS Lett.* 290:123–130.

Gebbink, M.F.B.G., G.C.M. Zondag, R.W. Wubbolts, R.L. Beijersbergen, I. van Etten, and W.H. Moolenaar. 1993. Cell–cell adhesion mediated by a receptor-like protein tyrosine phosphatase. *J. Biol. Chem*. 268:16101–16104.

Gebbink, M.F.B.G., G.C.M. Zondag, G.M. Koningstein, E. Feiken, R.W. Wubbolts, and W.H. Moolenaar. 1995. Cell surface expression of receptor protein tyrosine phosphatase RPTPμ is regulated by cell–cell contact. *J. Cell Biol*. 131:251–260.

Gershon, T.R., M.W. Baker, M. Nitabach, and E.R. Macagno. 1998. The leech receptor protein tyrosine phosphatase HmLAR2 is concentrated in growth cones and is involved in process outgrowth. *Development (Camb.)*. 125: 1183–1190.

Gumbiner, B.M. 1995. Signal transduction by β-catenin. *Curr. Opin. Cell Biol*. 7:634–640.

Halfter, W., D.F. Newgreen, J. Sauter, and U. Schwarz. 1983. Oriented axon outgrowth from avian embryonic retinae in culture. *Dev. Biol*. 95:56–64.

Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. pp60v-src causes tyrosine phosphorylation and inactivation of the N-cadherin catenin cell adhesion system. *EMBO (Eur. Mol. Biol. Organ.) J*. 12:307–314.

Hamburger, V., and H. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol*. 88:49–92.

Hatta, K., and M. Takeichi. 1986. Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature*. 320:447–449.

Hiscox, S., and W.G. Jiang. 1998. Association of PTPμ with catenins in cancer cells: a possible role for E-cadherin. *Int. J. Oncology*. 13:1077–1080.

Hoschuetzky, H., H. Aberle, and R. Kemler. 1994. β catenin mediates the interaction of the cadherin catenin complex with epidermal growth factor receptor. *J. Cell Biol*. 127:1375–1380.

Ignelzi, M.A., D.R. Miller, P. Soriano, and P.F. Maness. 1994. Impaired neurite outgrowth of src-minus cerebellar neurons on the cell adhesion molecule L1. *Neuron*. 12:873–884.

Inuzuka, H., S. Miyatani, and M. Takeichi. 1991. R-cadherin: a novel Ca^{2+} dependent cell–cell adhesion molecule expressed in the retina. *Neuron*. 7:69–79.

Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *TIG (Trends Genet.)*. 9:317–321.

Krueger, N.X., D. Van Vactor, H.I. Wan, W.M. Gelbart, C.S. Goodman, and H. Saito. 1996. The transmembrane tyrosine phosphatase DLAR controls motor axon guidance in *Drosophila*. *Cell*. 84:611–622.

Kypta, R., H. Su, and L. Reichardt. 1996. Association between a transmembrane protein tyrosine phosphatase and the cadherin-catenin complex. *J. Cell Biol*. 134:1519–1529.

Lagenaur, C., and V. Lemmon. 1987. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. *Proc. Natl. Acad. Sci. USA*. 84:7753–7757.

Lemmon, V., S.M. Burden, H.R. Payne, G.J. Elmslie, and M.L. Hlavin. 1992. Neurite growth on different substrates: permissive versus instructive influences and the role of adhesive strength. *J. Neurosci*. 12:818–826.

Lemmon, V., K. Farr, and C. Lagenaur. 1989. L1 mediated axon outgrowth occurs via a homophilic binding mechanism. *Neuron*. 2:1597–1603.

Lemmon, V., and S. McLoon. 1986. The appearance of an L1-like molecule in the chick primary visual pathway. *J. Neurosci*. 6:2987–2994.

Lin, C.H., and P. Forscher. 1993. Cytoskeletal remodeling during growth cone–target interactions. *J. Cell Biol*. 121:1369–1383.

Lochter, A., and M. Schachner. 1993. Tenascin and extracellular matrix glycoproteins: from promotion to polarization of neurite outgrowth in vitro. *J. Neurosci*. 13:3986–4000.

Matsunaga, M., K. Hatta, A. Nagafuchi, and M. Takeichi. 1988a. Guidance of optic nerve fibres by N-cadherin adhesion molecules. *Nature*. 334:62–64.

Matsunaga, M., K. Hatta, and M. Takeichi. 1988b. Role of N-cadherin cell adhesion molecules in the histogenesis of neural retina. *Neuron*. 1:289–295.

Matsuyoshi, N., M. Hamaguchi, S. Taniguchi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1992. Cadherin-mediated cell–cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol*. 118:703–714.

Mey, J., and S. Thanos. 1992. Development of the visual system of the chick—a review. *J. Hirnforsch*. 33:673–702.

Mi, Z., W. Weng, M.H. Hankin, V. Narayanan, and C.F. Lagenaur. 1998. Maturational changes in cell surface antigen expression in the mouse retina and optic pathway. *Dev. Brain Res*. 106:145–154.

Neel, B.G., and N.K. Tonks. 1997. Protein tyrosine phosphatases in signal transduction. *Curr. Opin. Cell Biol*. 9:193–204.

Neugebauer, K.M., C.J. Emmett, K.A. Venstrom, and L.F. Reichardt. 1991. Vitronectin and thrombospondin promote retinal neurite outgrowth; developmental regulation and role of integrins. *Neuron*. 6:345–358.

Ochiai, A., S. Akimoto, Y. Kanai, T. Shibata, T. Oyama, and S. Hirohashi. 1994. c-erbB-2 gene product associates with catenins in human cancer cells. *Biochem. Biophys. Res. Commun*. 205:73–78.

Paulus, W., I. Baur, F.M. Boyce, X.O. Breakfield, and S.A. Reeves. 1996. Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *J. Virol*. 70:62–67.

Prada, C., J. Puga, L. Perez-Mendez, R. Lopez, and G. Ramirez. 1991. Spatial and temporal patterns of neurogenesis in the chick retina. *Eur. J. Neurosci*. 3:559–569.

Redies, C., and M. Takeichi. 1993. N- and R-cadherin expression in the optic nerve of the chicken embryo. *Glia*. 8:161–171.

Reichardt, L.F., B. Bossy, S. Carbonetto, I. de Curtis, C. Emmett, D.E. Hall, M.J. Ignatius, F. Lefcort, E. Napolitano, T. Large, et al. 1990. Neuronal receptors that regulate axon growth. *In* Cold Spring Harbor Symposia on Quantitative Biology. Vol. 55. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 341–350.

Reichardt, L.F., and K.J. Tomaselli. 1991. Extracellular matrix molecules and their receptors: functions in neural development. *Annu. Rev. Neurosci*. 14: 531–570.

Rutishauser, U. 1983. Molecular and biological properties of a neural cell adhesion molecule. *Cold Spring Harbor Symp. Quant. Biol*. 48:501–514.

Rutishauser, U., J.-P. Thiery, R. Brackenbury, and G.M. Edelman. 1978. Adhesion among neural cells of the chick embryo. III. Relationship of the surface molecule CAM to cell adhesion and the development of histotypic patterns. *J. Cell Biol*. 79:371–381.

Serra-Pages, C., H. Saito, and M. Streuli. 1994. Mutational analysis of proprotein processing, subunit association, and shedding of the LAR transmembrane protein tyrosine phosphatase. *J. Biol. Chem*. 269:23632–23641.

Shibamoto, S., M. Hayakawa, K. Takeuchi, T. Hori, N. Oku, K. Miyazawa, N. Kitamura, M. Takeichi, and F. Ito. 1994. Tyrosine phosphorylation of β catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhes. Commun*. 1:295–305.

Silver, J., and U. Rutishauser. 1984. Guidance of optic axons in vivo by a preformed adhesive pathway on neuroepithelial endfeet. *Dev. Biol*. 106:485–499.

Sommer, L., M. Rao, and D.J. Anderson. 1997. RPTPδ and the novel protein tyrosine phosphatase RPTPψ are expressed in restricted regions of the developing nervous system. *Dev. Dyn*. 208:48–61.

Streuli, M. 1996. Protein tyrosine phosphatases in signaling. *Curr. Opin. Cell Biol*. 8:182–188.

Streuli, M., N. Krueger, P. Ariniello, M. Tang, J. Munro, W. Blattler, D. Adler, C. Disteche, and H. Saito. 1992. Expression of the receptor-linked protein tyrosine phosphatase LAR: proteolytic cleavage and shedding of the CAM-like extracellular region. *EMBO (Eur. Mol. Biol. Organ.) J*. 11:897–907.

Tang, J., L. Landmesser, and U. Rutishauser. 1992. Polysialic acid influences specific pathfinding by avian motoneurons. *Neuron*. 8:1031–1044.

Tessier-Lavigne, M. 1995. Eph receptor tyrosine kinases, axon repulsion, and the development of topographic maps. *Cell*. 82:345–348.

Tessier-Lavigne, M., and C.S. Goodman. 1996. The molecular biology of axon guidance. *Science*. 274:1123–1133.

Tsui, H.T., W.B. Pope, C.S. Kim, and W.L. Klein. 1992. Transient expression of adheron molecules during chick retinal development. *J. Neurobiol*. 23:720–738.

Tsukita, S., K. Oishi, T. Akiyama, Y. Yamanashi, T. Yamamoto, and S. Tsukita. 1991. Specific proto-oncogene tyrosine kinases of *src* family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. *J. Cell Biol*. 113:867–879.

Volberg, T., B. Geiger, R. Dror, and Y. Zick. 1991. Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells. *Cell Regul*. 2:105–120.

Volberg, T., Y. Zick, R. Dror, I. Sabanay, C. Gilon, A. Levitzki, and B. Geiger. 1992. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO (Eur. Mol. Biol. Organ.) J*. 11:1733–1742.

Warren, S.L., and W.J. Nelson. 1987. Nonmitogenic morphoregulatory action of pp60v-src on multicellular epithelial structures. *Mol. Cell. Biol*. 7:1326–1337.

Zondag, G., G. Koningstein, Y.P. Jiang, J. Sap, W.H. Moolenaar, and M. Gebbink. 1995. Homophilic interactions mediated by receptor tyrosine phosphatases μ and κ. *J. Biol. Chem*. 270:14247–14250.