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The Carbonic Anhydrase Domain of Receptor Tyrosine Phosphatase β Is a Functional Ligand for the Axonal Cell Recognition Molecule Contactin

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Summary

Receptor-type protein tyrosine phosphatase β (RPTPβ) is expressed in the developing nervous system and contains a carbonic anhydrase (CAH) domain as well as a fibronectin type III repeat in its extracellular domain. Fusion proteins containing these domains were used to search for ligands of RPTPβ. The CAH domain bound specifically to a 140 kDa protein expressed on the surface of neuronal cells. Expression cloning in COS7 cells revealed that this protein is contactin, a GPI membrane-anchored neuronal cell recognition molecule. The CAH domain of RPTPβ induced cell adhesion and neurite growth of primary tectal neurons, and differentiation of neuroblastoma cells. These responses were blocked by antibodies against contactin, demonstrating that contactin is a neuronal receptor for RPTPβ. These experiments show that an individual domain of RPTPβ acts as a functional ligand for the neuronal receptor contactin. The interaction between contactin and RPTPβ may generate unidirectional or bidirectional signals during neural development.

Introduction

The ability of cells to respond to signals from their microenvironment is a fundamental feature of development. In the developing nervous system, neurons migrate and extend axons to establish their intricate network of synaptic connections (Goodman and Shatz, 1993). During migration and axonal pathfinding, cells are guided by both attractive and repulsive signals (Hynes and Lander, 1992; Keynes and Cook, 1992). The ability of the neuron to respond to these signals requires cell surface molecules that are able to receive the signals and to transmit them to the cell interior, resulting in specific biological responses.

It is well established that protein tyrosine phosphorylation is responsible for the regulation of many cellular responses to external stimuli crucial for cell growth, proliferation, and differentiation (Schlessinger and Ullrich, 1992). Tyrosine phosphorylation was implicated in several developmental processes in the nervous system. For example, receptor tyrosine kinases were shown to affect neuronal survival (Chao, 1992) and cell fate determination (Zipursky and Rubin, 1994). Nonreceptor tyrosine kinases were shown to be downstream elements in signaling via cell recognition molecules that play a role in cell guidance and migration (Ignelzi et al., 1994; Umemori et al., 1994).

The transient nature of signaling by phosphorylation requires specific phosphatases for control and regulation (Hunter, 1995). Indeed, many protein tyrosine phosphatases were shown to be expressed in specific regions of the developing brain, including the olfactory neuroepithelium (Walton et al., 1993), the cortex (Sahin et al., 1995), and retinal Müller glia (Shock et al., 1995). Furthermore, expression of several tyrosine phosphatases, such as protein-tyrosine phosphatase α (PTPα) (den Hertog et al., 1993), PC12-PTP1 (Sharama and Lombroso, 1995), and several forms of leukocyte common antigen–related (LAR) tyrosine phosphatase (Zhang and Longo, 1995) were found to be regulated during neuronal differentiation of P19 or PC12 cells.

Receptor-type protein tyrosine phosphatases (RPTPs) were subdivided into several groups on the basis of structural characteristics of their extracellular domains (Charbonneau and Tonks, 1992; Barnea et al., 1993). RPTPβ (also known as RPTPζ) and RPTPγ are members of a distinct group of phosphatases characterized by the presence of carbonic anhydrase (CAH)-like domains, fibronectin type III (FNIII) repeats, and a long cysteine-free region (spacer domain) in their extracellular domain (Barnea et al., 1993; Krueger and Saito, 1992; Levy et al., 1993). The expression of RPTPβ is restricted to the central and peripheral nervous system, while RPTPγ is expressed both in the developing nervous system and in a variety of other tissues in the adult rat (Canoll et al., 1993; Barnea et al., 1993). RPTPβ exists in three forms, one secreted and two membrane bound, that differ by the absence of 860 residues from the spacer domain (Levy et al., 1993; Maurel et al., 1994). The secreted form was identified in rat brain as a chondroitin sulfate proteoglycan called phosphacan (3F8 proteoglycan) (Barnea et al., 1994a; Maurel et al., 1994; Shitara et al., 1994). The transmembrane form was also shown to be expressed in a form of a chondroitin sulfate proteoglycan (Barnea et al., 1994b). It is presently unknown whether the glycosaminoglycan attachment sites are utilized only in the insert of 860 amino acids that is not present in the short form of RPTPβ. It was shown that purified phosphacan can interact in vitro with the extracellular matrix protein tenascin and with the adhesion molecules N-CAM and Ng-CAM (for neural cell adhesion molecule and neuron–glia cell adhesion molecule, respectively) (Barnea et al., 1994b; Grumet et al., 1993, 1994; Milev et al., 1994).

In the work presented in this report, we used fusion proteins containing the CAH domain of RPTPβ as probes for the identification, purification, and cloning of a protein that bound specifically to RPTPβ. Using expression cloning in COS7 cells and affinity chromatography, we show that the CAH domain of RPTPβ binds specifically to the cell recognition molecule contactin (F11/F3). Moreover, we

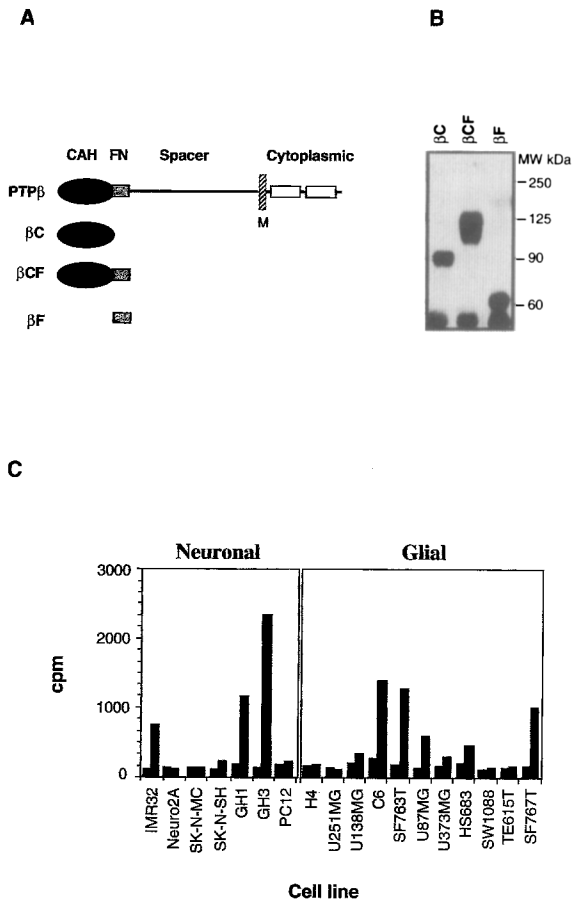

Figure 1. Detection of Cell Surface–Bound Ligands for RPTPβ

(A) Schematic presentation of RPTPβ and different subdomains used to construct fusion proteins with human IgG1–Fc. CAH, CAH-like domain; FN, FNIII repeat; M, transmembrane domain.

(B) Expression of the chimeric IgG molecules. Different β fusion proteins containing the CAH or the FNIII domains (βC and βF respectively) or both domains (βCF) were purified, separated on SDS gel, and immunoblotted with antibodies against human IgG.

(C) Binding of βCF to a cell line derived from neuronal (neuroblastoma and neuroendocrine) and glial (glioblastoma and astrocytoma) tumors. Cells were incubated with control medium (closed bars) or with medium containing βCF fusion protein at 0.4 μg/ml (βCF; stippled bars). Bound βCF was detected by incubation with ^{125}I–protein A as described in Experimental Procedures.

demonstrate that the CAH domain of RPTPβ functions as a ligand for contactin. The interactions between a cell recognition molecule and a transmembrane protein tyrosine phosphatase may play an important role during neural development and differentiation.

Results

Membrane-Bound Ligands for RPTPβ Are Differentially Expressed in Neuronal and Glial Cell Lines

To identify cellular ligands for RPTPβ, we have constructed fusion proteins containing different subdomains of RPTPβ and the Fc portion of human immunoglobulin G (IgG). Three chimeric constructs were generated, one containing both the CAH and the FNIII domains (βCF) and two others carrying each domain alone (βC or βF; Figures 1A and 1B). We first used βCF to screen for membrane-bound ligands on the surface of different neuronal and glial cell lines. As shown in Figure 1C, several cell lines were identified that specifically interact with this fusion protein, including IMR32 neuroblastoma cells, the two closely related neuroendocrine-derived cell lines GH3 and GH1, and five different glioblastoma cell lines.

The finding that the positive cell lines were derived from both glial and neuronal origins raised the possibility that RPTPβ may interact with two different membrane-associated ligands. Alternatively, the same ligand may be expressed on the cell surface of both neurons and glial cells. To explore these possibilities, we have examined the binding properties of fusion proteins that contain either the CAH domain (βC) or the FNIII domain (βF) of RPTPβ. The results presented in Figure 2A show that the βC fusion protein bound only to neuronal and neuroendocrine cell lines but not to glioblastoma cells. These experiments demonstrated that the CAH domain is responsible for binding to neuronal cells, while the FNIII repeat is required for binding to glial cells. Accordingly, if the binding of βC to a neuronal ligand reflects the interactions that take place in vivo, one would expect to find similar binding of the CAH domain to primary neurons. We therefore analyzed the binding of the different fusion proteins to cultured dorsal root ganglion cells (DRGs), followed by detection of the bound proteins by immunostaining. The results presented in Figure 2B show that βC and βCF bound to GH3 cells, as well as to primary neurons. A fusion protein containing the FNIII domain alone (βF) did not bind either to GH3 cells or to DRG neurons. In other experiments, we detected binding of βF to several glial cell lines, but could not detect binding of the FNIII repeat to neuronal-derived cell lines or neurons derived from rat DRGs or from chick cortex (data not shown). In addition, we also examined whether the binding specificity observed with the CAH domain of RPTPβ is unique to this receptor by analyzing the binding of a fusion protein containing the CAH domain of RPTPγ (Barnea et al., 1993). This experiment showed that the CAH domain of RPTPγ does not bind either to GH3 cells or to primary neurons (Figure 2C; data not shown).

All together, these results suggested that specific ligands for RPTPβ exist on the surface of cells from neuronal and glial origin. Moreover, different subdomains of the receptor appear to mediate interactions with different ligands. On the basis of this analysis, we concluded that the CAH domain of RPTPβ mediates interactions with neurons, while the FNIII domain is responsible for interactions with glial cells. In the work presented in this report, we describe the identification, purification, and molecular characterization of the ligand for the CAH domain of RPTPβ.

Covalent Cross-Linking Experiments Reveal a 140 kDa Protein That Interacts with the CAH Domain of RPTPβ

To characterize ligands for RPTPβ, we used a reversible cross-linking agent, 3,3′-dithiobis(sulfosuccinimidylpropi-

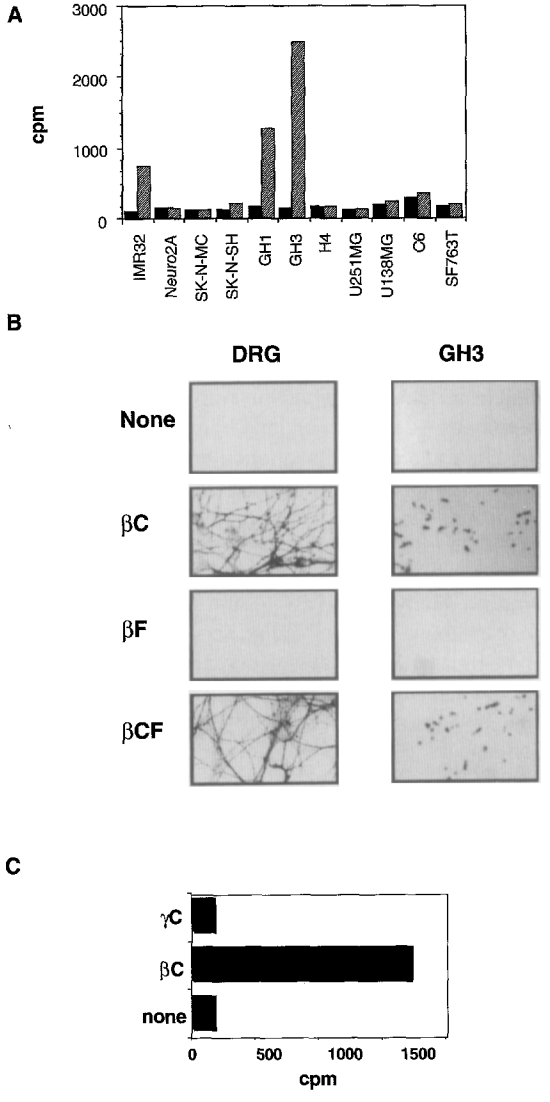

Figure 2. Specific Binding of the CAH-like Domain of RPTPβ to Neurons and Neuronal Cell Lines

(A) Binding of βC to neuronal and glial tumor-derived cell lines. Control medium (none; closed bars) or medium containing βC–Fc fusion protein at 0.25 μg/ml was used (βC; stippled bars). Bound fusion proteins were detected by incubation with ^{125}I–protein A and determination of radioactive content.

(B) Binding of the different subdomains of RPTPβ to GH3 cells and to primary neurons. GH3 cells and primary cultures of rat DRGs were incubated with control medium (none) or medium containing a fusion protein with the CAH domain (βC), the FNIII domain (βF), or a fusion protein containing both domains (βCF) for 1 hr at room temperature. Unbound proteins were removed, and the bound Fc fusion proteins were visualized by immunostaining with biotinylated anti-human IgG antibodies and streptavidin–alkaline phosphatase as described in Experimental Procedures.

(C) Fc fusion proteins containing the CAH domain of RPTPβ (βC) or the CAH domain of RPTPγ (γC) were applied in binding assay to GH3 cells. Binding was performed as described in (A).

onate) (DTSSP), and searched for proteins that specifically bound to βC. Two of the cell lines that bound βC (IMR32 and GH3), as well as COS7 cells as a control, were first allowed to react with the fusion proteins containing either

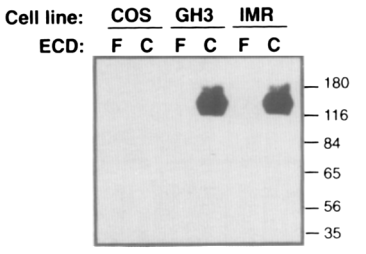

Figure 3. Covalent Cross-Linking of βC–Fc to a Cell Membrane–Bound Ligand

Fc fusion proteins containing the CAH domain (C) or the FN III domain (F) of RPTPβ were allowed to bind to [^{35}S]methionine-labeled IMR32 neuroblastoma cells (IMR), GH3 pituitary tumor cells, or COS7 cells as indicated. Following a 1 hr incubation, unbound proteins were washed away, and 1 mM of the reversible covalent cross-linking agent (DTSSP) was added for an additional 30 min at 4°C. Cell lysates were prepared, and the cross-linked proteins were precipitated with protein A–Sepharose as detailed in Experimental Procedures. The proteins were resolved on 8%–16% SDS gels under reducing conditions, followed by autoradiography. Under these conditions, the cross-linker is cleaved. Molecular mass marker proteins are indicated in kilodaltons.

the FNIII or the CAH domains, and then complexes were cross-linked and precipitated. In an effort to identify the true molecular mass of the putative ligand, we utilized the covalent cross-linking agent DTSSP, which undergoes cleavage in reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) conditions. As shown in Figure 3, a protein with an apparent molecular mass of 140 kDa specifically reacted with βC in rat GH3 and human IMR32 cells. No reactivity was detected in control cells or in cells incubated with βF. This result suggested that a similar ligand is expressed on the surface of rat GH3 and human IMR32 cells.

Molecular Cloning of a Candidate Ligand for RPTPβ from Rat GH3 Cells

To clone the gene that encodes the 140 kDa candidate ligand, we employed an expression cloning strategy. Plasmid pools made from a GH3 cDNA library were transfected into COS7 cells that were subsequently screened for their ability to bind βCF. Positive cells were detected by immunostaining with biotinylated anti-human IgG antibodies and streptavidin–alkaline phosphatase. One plasmid pool was identified that when transfected into cells resulted in several positively stained cells (Figure 4A). This pool was subdivided and rescreened four times, until a single clone (F8) was isolated. Transfection of COS7 cells with this plasmid resulted in positive staining of approximately 25%–50% of the cells. DNA sequence analyses of clone F8 showed that it contained a 4.0 kb insert and a single open reading frame of 3063 nt. The deduced 1021 amino acid sequence encoded by this clone is presented in Figure 4B. Database searches with this sequence showed that it shares 95% and 99% identity with the amino acid sequences of human and mouse contactin (F11/F3), respectively (Berglund and Ranscht, 1994; Gennarini et al., 1989; Reid et al., 1994). We therefore concluded that the ligand for RPTPβ cloned from GH3 cells is the rat homolog of contactin. This protein consists of six C2-type immunoglobulin domains, four FNIII repeats, and a hydrophobic

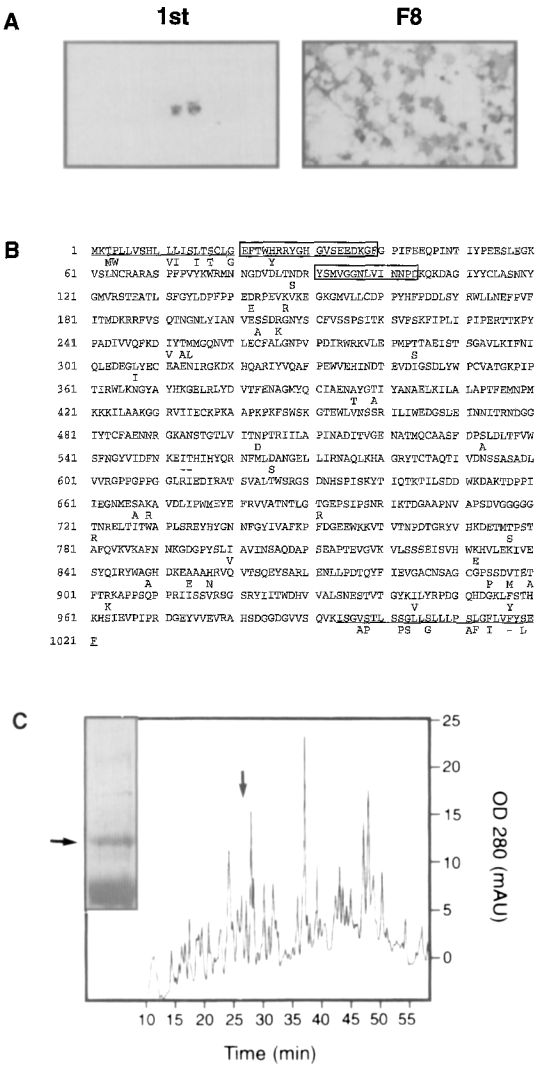

Figure 4. Expression Cloning and Purification of the Ligand for the CAH Domain of RPTPβ

(A) Screening of the GH3 expression library in COS cells. COS7 cells were transfected with a cDNA pool consisting of 3000 independent clones (first round of screening) or with clone F8, which was isolated after the fifth round of screening (F8). Binding of βCF was detected by immunostaining as described in Experimental Procedures.

(B) Complete amino acid sequence of the ligand of the CAH domain. The deduced amino acid sequence of the F8 cDNA clone. The hydrophobic sequences in the N- and C-terminal regions are underlined, and the partial amino acid sequences obtained from p140 purified by affinity chromatography are boxed. Residues that are different from human contactin are shown below.

(C) Affinity purification of p140 from GH3 cells. p140 was purified from solubilized GH3 membranes by using a βCF column. The sample was loaded on 7.5% SDS gel, transferred to ProBlott membranes, and stained with Coomassie blue (inset). One quarter of the purified p140 was excised from the gel and used for determination of the N-terminal sequence. The remaining material was digested with trypsin and loaded on Reliasil C-18 column. The HPLC profile is shown, and the peptide whose sequence was determined is marked by an arrow. The two partial sequences are identical to those that are boxed in Figure 4B.

region that mediates its attachment to the membrane by a glycosylphosphatidylinositol (GPI) linkage (Figure 4B; Gennarini et al., 1989; Reid et al., 1994). Functionally, contactin is a neural cell adhesion receptor that is thought to play a morphogenic role during the development of the nervous system (Walsh and Doherty, 1991).

In parallel to the expression cloning strategy, we used a biochemical approach that utilized the CAH domain as an affinity reagent for protein purification. The 140 kDa protein was purified from solubilized membranes prepared from GH3 cells by using an affinity column of βCF (Figure 4C). After resolution of the eluted 140 kDa protein on SDS–PAGE, it was subjected to N-terminal sequencing either directly or following digestion with trypsin. Two peptide sequences were obtained, one from the N-terminus of the protein, and a second sequence from a tryptic fragment. Both sequences matched the translated F8 sequence (see Figure 4B) and confirmed that the 140 kDa ligand for the CAH domain of RPTPβ is contactin.

Binding Analysis of RPTPβ and Contactin

We next examined the binding specificity of different subdomains of RPTPβ toward contactin. COS7 cells were transfected with rat contactin (clone F8) and analyzed for their ability to bind fusion proteins containing the CAH domain, the FNIII domain, or both (Figure 5A). As expected, expression of contactin enabled the binding of the CAH domain of RPTPβ to the cells. The FNIII domain alone did not bind to contactin-expressing cells. In addition, we obtained similar results with a fusion protein that carries most of the extracellular region of the short form of RPTPβ (amino acids 1–644; data not shown).

We next explored the reciprocal interaction, namely, whether soluble contactin molecules were able to bind specifically to cells expressing RPTPβ. In these experiments, COS7 cells were transfected with chimeric receptor constructs that consist of the entire extracellular region of the short form of RPTPβ (βCFS–EK), the CAH domain plus the FNIII repeat (βCF–EK), or the CAH domain alone (βC–EK) fused to the transmembrane and intracellular domains of the EGF receptor kinase (EK). We used a chimeric receptor instead of the wild-type phosphatase, since we were not able to express the wild-type phosphatase in heterologous cells. The experiment presented in Figure 5B shows that human contactin–Fc fusion protein binds to cells transfected with these chimeric receptors but not to control cells. Taken together, these results demonstrate that expression of contactin is both necessary and sufficient for binding to the CAH domain RPTPβ.

Soluble Contactin Released from the Membrane by Phospholipase C Treatment Interacts with RPTPβ

Contactin belongs to a family of recognition molecules that are anchored to the plasma membrane via GPI. It was, therefore, of interest to examine the effect of phosphatidylinositol-specific phospholipase C (PI-PLC) treatment on the interaction between contactin and RPTPβ. PI-PLC treatment completely abolished the binding of βCF to

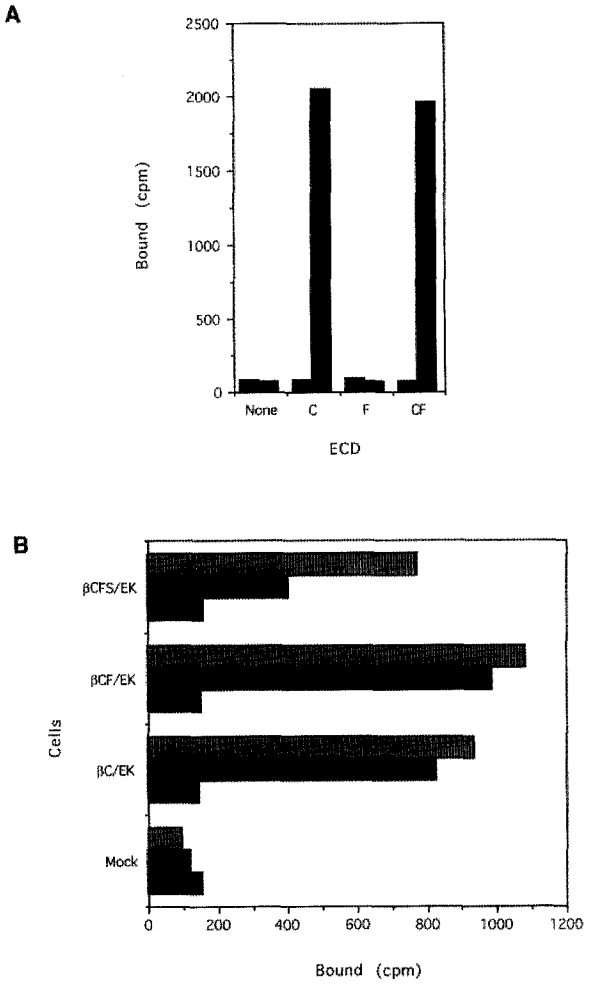

Figure 5. Binding of Soluble RPTPβ Forms to Contactin and Binding of a Soluble Contactin Form to RPTPβ

(A) Binding specificity of subdomains of RPTPβ to contactin-expressing cells. COS cells were transfected with clone F8 encoding wild-type contactin (stippled bars) or with β-gal-expressing plasmid as a control (closed bars). Cells were analyzed for their ability to bind Fc fusion proteins containing the CAH domain (C), the FNIII domain (F), or both (CF).

(B) Binding of human contactin–Fc fusion protein to cells expressing RPTPβ–EGF receptor chimeras. COS cells were transfected with chimeric molecules in which the extracellular region of the short form of RPTPβ (βCFS–EK), the CAH, and the FNIII domains (βCF–EK) or the CAH domain alone (βC–EK) were fused to the transmembrane and cytoplasmic domains of the EGF receptor. Binding of the contactin–Fc fusion protein (stippled bars) or control Fc fusion protein (closed bars) was carried on after 72 hr as described in (A). The expression level of the chimeric receptors was determined by using antibodies against the extracellular domain of human RPTPβ (shaded bars) as described in Experimental Procedures.

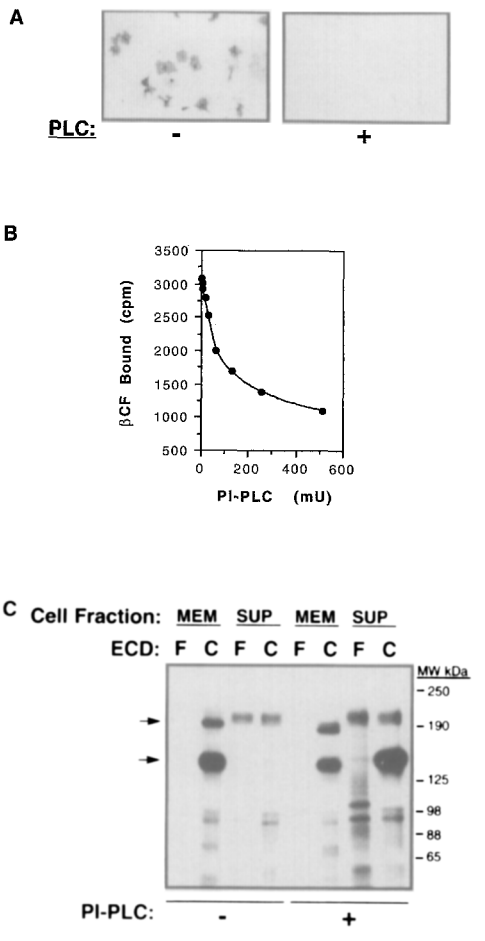

Figure 6. Release of Surface-Bound Contactin by PI-PLC Treatment

(A) COS7 cells were transfected with expression vector that directs the synthesis of wild-type contactin, and 72 hr later, the cells were treated with (plus) or without (minus) PI-PLC (400 mU). Cells were incubated with βCF and positive cells detected as described in the legend to Figure 4.

(B) GH3 cells were treated with increasing amounts of PI-PLC as indicated and then incubated with medium containing βCF at 0.4 μg/ml for 1 hr. After extensive washing, cell-bound proteins were detected by incubation with ^{125}I–protein A.

(C) GH3 cells were metabolically labeled with [^{35}S]methionine and treated with or without PI-PLC as indicated. Cell supernatants (SUP) and solubilized cell membranes (MEM) were subjected to affinity precipitation using Fc fusion proteins containing the CAH domain (C) or the FNIII repeat (F) of RPTPβ. The washed complexes were separated on a 7.5% SDS gel under reducing conditions. An autoradiogram of the fixed and dried gel is shown along with the location of molecular mass marker proteins. The 140 kDa band of contactin and the coprecipitated 180 kDa protein are marked by arrows.

COS7 cells expressing contactin (Figure 6A). Similar results were obtained with GH3 cells, although in this case not all binding sites were removed by PI-PLC treatment (Figure 6B). We do not know whether the incomplete release of contactin from GH3 cells is due to the presence of PLC-resistant binding sites, is a consequence of interactions with other proteins (see below), or is due to incomplete cleavage.

Like other GPI-linked proteins, contactin exists in both membrane-bound and soluble forms (Théveniau et al., 1992). We examined whether different forms of contactin, including those released by PI-PLC treatment, are able to bind to RPTPβ. Metabolically labeled GH3 cells were treated with PI-PLC, and contactin was purified by bioaffinity precipitation either from membrane preparations or from cell supernatants (Figure 6C). Two proteins (p140/contactin and p180) were found to be associated with βC in the membrane fractions in the absence of PI-PLC treat-

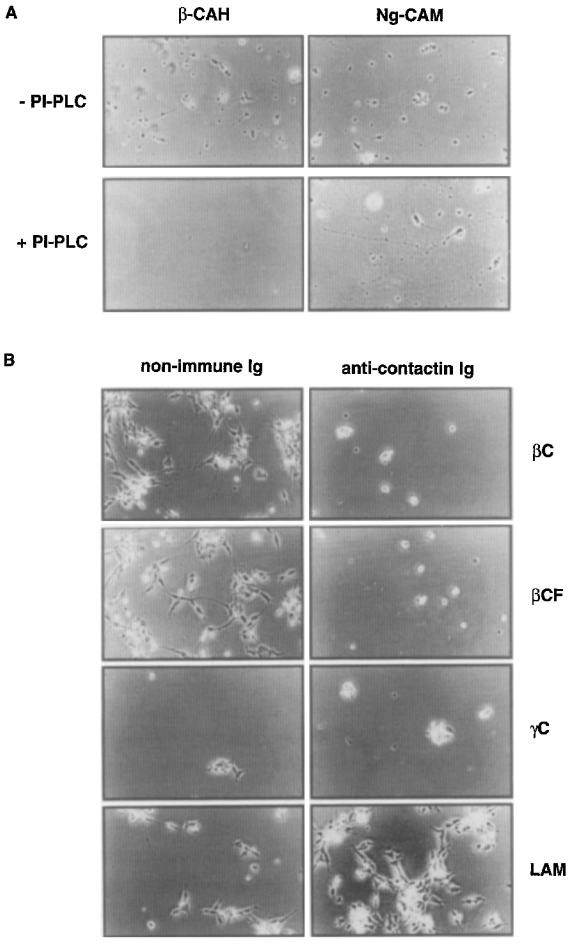

Figure 7. Biological Effect of the CAH Domain on Neurite Outgrowth and Cell Differentiation

(A) Effect of PI-PLC on neurite growth of chick neurons on dishes coated with the CAH domain of RPTPβ. Primary tectal cells were treated with or without PI-PLC as indicated for 1 hr and plated on βCF- or Ng-CAM-coated dishes. After 24 hr, unbound cells were removed by gentle wash, and the plate was fixed and photographed.

(B) Process extension of IMR32 cells induced by βC and βCF fusion proteins is inhibited by antibodies against contactin. Dishes were coated in a circular array with 2 μl drops of Fc fusion proteins (βC, βCF, and γC) or with laminin (LAM). After removal of the unabsorbed proteins, the dishes were incubated with bovine serum albumin, and the IMR32 cells were allowed to adhere to the dishes for 3 hr. The medium was removed and replaced with fresh medium containing nonimmune or anti-contactin immunoglobulin antibodies as indicated, at a final concentration of 250 μg/ml. The cultures were incubated for 48 hr, fixed, and photographed as described in Experimental Procedures (magnification, 92×).

ment. Neither protein was present in the supernatant, nor did they interact with the FNIII domain of RPTPβ. However, after PI-PLC treatment, we were able to precipitate soluble contactin from the medium of the cells with a fusion protein containing the CAH domain, indicating that the soluble form generated by phospholipase treatment is still able to interact with RPTPβ. In contrast, PI-PLC treatment did not release the 180 kDa protein from the cells, suggesting that it may be a contactin-associated integral membrane protein. Since contactin by itself binds to the CAH domain, the p180 protein is probably a contactin-bound protein isolated by the affinity procedure. It is, however, possible that this protein may interact directly with RPTPβ. An intriguing possibility is that p180 is a component of the contactin signaling complex in neurons (see below).

Neurite Outgrowth Induced by the CAH Domain of RPTPβ Is Mediated through Contactin

Contactin was shown to be involved in both positive and negative responses of neurons to various stimuli (Brümmendorf and Rathjen, 1993). When presented as a ligand to neurons, in either membrane-bound or soluble forms, contactin induces axonal growth (Brümmendorf et al., 1993; Durbec et al., 1992; Gennarini et al., 1989). It was suggested that this response is mediated by Nr-CAM (for Ng-CAM-related CAM) (Morales et al., 1993). Other studies demonstrated that the extracellular protein janusin (restrictin) was able to bind to contactin, leading to neuronal repulsion (Pesheva et al., 1993). The results presented in this report show that the CAH domain of RPTPβ binds specifically to contactin expressed on the surface of neuronal cells. To analyze the biological effects of this binding, chick tectal cells, which express contactin on their cell surfaces, were plated on dishes coated with βCF fusion protein or with Ng-CAM as controls. These cells attached and extended processes on either substrate (Figure 7A). Treatment of the cells with PI-PLC prior to plating completely abolished cell attachment and neurite extension when βCF was used as the substrate. Similar results were obtained with βC as a substrate (data not shown). However, PI-PLC treatment had no effect on cells growing on either Ng-CAM or laminin (Figure 7A; data not shown). We concluded that the CAH domain of RPTPβ is a permissive substrate for neuronal adhesion and neurite growth and that cell adhesion and axonal elongation induced by the CAH domain are mediated through a GPI-anchored receptor.

We next investigated whether contactin is a functional neuronal receptor for the CAH domain of RPTPβ. In these experiments, we used the human neuroblastoma cell line IMR32, which has the capacity to differentiate and to elaborate neurites in response to different stimuli (Lüdecke and Unnsicker, 1990). These cells have fibroblastic morphology when grown on cell culture dishes coated with fibronectin, but assume a neuronal phenotype and extend processes with growth cones when plated on laminin (Figure 7B; data not shown). A similar morphologic differentiation was seen after plating the cells on a substrate composed of the CAH domain of RPTPβ. In contrast, the CAH domain of RPTPγ did not promote cell adhesion and differentiation. We next examined the effects of antibodies against contactin on the differentiation of these cells induced by different substrates. The experiment presented in Figure 7B shows that antibodies against contactin inhibited the differentiation of IMR32 cells grown on βC and βCF substrates but had no effect on differentiation of cells grown on a laminin substrate (Figure 7B). In the presence of these antibodies, the IMR32 cells also retracted their processes, and many cells lifted off the dish, yielding fewer cells after 2 days of incubation. No effect was observed

with control antibodies. Thus, neurite growth and differentiation induced by the CAH domain of RPTPβ is mediated by contactin molecules expressed on the surface of these cells.

Discussion

During development of the nervous system, neurons are guided by secreted and cell-bound molecules that provide both negative and positive cues. In this report, we show that RPTPβ, a receptor-type protein-tyrosine phosphatase, may provide such a signal by interacting with the axonal recognition molecule contactin. RPTPβ is a developmentally regulated protein that exists in three forms, one secreted and two membrane bound. The extracellular region of RPTPβ has a multidomain structure consisting of a CAH-like domain, a single FNIII repeat, and a long cysteine-free spacer region. The complex structural nature of its extracellular region may result in a multifunctional protein that is able to interact with different proteins. Indeed, we found that the CAH and the FNIII domains bind to at least two potential ligands present on neurons and glial cells. Functional expression cloning in COS7 cells and affinity purification with a specific affinity matrix followed by microsequencing enabled unequivocal identification of the cell recognition molecule contactin (F3/F11) as a neuronal ligand of RPTPβ. The interaction between contactin and RPTPβ is mediated via the CAH domain of the phosphatase, while the FNIII domain appears to bind to another molecule expressed on the surface of glial cells. It was previously shown that the secreted proteoglycan form of RPTPβ interacts with tenascin, N-CAM, and Ng-CAM (Grumet et al., 1993, 1994; Barnea et al., 1994b; Milev et al., 1994). Since these proteins do not bind directly to the CAH or the FNIII domain of RPTPβ (data not shown), they may interact with the large spacer domain of the phosphatase. Alternatively, they could interact with RPTPβ through a third component. Contactin may fulfill this function, since it has been shown to interact with Ng-CAM, Nr-CAM, and the matrix proteins tenascin and restrictin (Brümmendorf et al., 1993; Morales et al., 1993; Zisch et al., 1992). It appears that the various subdomains of the extracellular region of RPTPβ are able to interact with several distinct proteins that are expressed on diverse cell types in the central nervous system.

In contrast with other cell recognition molecules that are widely expressed in the nervous system, members of the contactin subgroup appear to be expressed in a restricted manner on specific axons during development (Dodd et al., 1988; Faivre-Sarrailh et al., 1992). The spatial and temporal expression pattern of these proteins suggests that they play an important role during development of the nervous system. Contactin was found to be exclusively expressed on neurons during development in fiber-rich areas of the retina, tectum, spinal cord, and cerebellum (Ranscht, 1988). It was found to be localized in the postnatal and adult mouse cerebellum in axonal extensions of the granule cells in the parallel layer (Faivre-Sarrailh et al., 1992). This pattern of expression is overlapping with the expression pattern of RPTPβ in the rat. RPTPβ was shown to be expressed in fiber-rich regions such as the parallel fibers of the cerebellum and the spinal cord (Canoll et al., 1993; Milev et al., 1994). RPTPβ is also expressed on glial and radial glial cells, and its secreted form is produced by astrocytes. It is possible, therefore, that both forms of RPTPβ could modulate neuronal function via interactions with contactin.

The contactin subgroup of glycoproteins all share structural similarity in that they are GPI-anchored proteins. They also exist in soluble forms generated as a result of membrane release or by expression of alternatively spliced forms (Brümmendorf and Rathjen, 1993). Differential expression of the membrane-bound and soluble forms of contactin was found in the hypothalamus–hypophyseal system (Rougon et al., 1994). RPTPβ also exists in membrane-bound or secreted forms that are developmentally regulated. It is possible, therefore, that RPTPβ and contactin can act both as ligand and receptor for each other. Hence, the classical notion of ligand–receptor interaction is inappropriate in this system, since the components might switch roles at different stages of development. For example, the soluble form of RPTPβ produced by glial cells may act as a ligand for the membrane-bound form of contactin expressed on the surface of neuronal cells. Conversely, the soluble form of contactin may act as ligand for the membrane-bound form of RPTPβ expressed on the surface of glial cells. Moreover, interaction between the membrane-bound forms of contactin expressed on the surface of neurons with the membrane-bound form of RPTPβ expressed on the surface of glial cells may lead to bidirectional signals between these two cell types. Such complex interactions between the various forms of RPTPβ and contactin may generate developmentally regulated unidirectional and bidirectional signals.

How does the CAH domain of RPTPβ bind to contactin? Carbonic anhydrases are highly efficient enzymes that catalyze the hydration of CO_2. It has previously been proposed that the CAH domain of PTPases is not endowed with enzymatic activity, owing to substitution of two of the three key histidine residues that are essential for enzymatic activity (Krueger and Saito, 1992; Barnea et al., 1993). However, the highly packed hydrophobic core, as well as the hydrophobic residues that are exposed on the surface of CAH structure and are conserved in the CAH domains of RPTPγ and RPTPβ, may be involved in protein–protein interaction and thus may function as a ligand-binding domain (Barnea et al., 1993). It is interesting to note that vaccinia virus contains a transmembrane protein with a CAH-like domain in its extracellular domain, which was thought to be involved in binding of the virion to host proteins (Maa et al., 1990). It is therefore possible that the CAH domains of RPTPγ and vaccinia virus are able to bind to other members of the contactin family of glycoproteins and that a contactin-related protein may function as a receptor for vaccinia virus.

How does contactin, a GPI-linked protein that is inserted into the outer leaflet of the plasma membrane, transmit a signal into the cells to promote neurite outgrowth? One possibility is that contactin is able to interact with a transmembrane signaling component. The p180 protein that

was coprecipitated with contactin is a candidate for such a signaling protein (Figure 6C). p180 is probably membrane associated, since it could not be released by phospholipase C treatment. Another potential signal transducer could be L1/Ng-CAM or a related molecule. This transmembrane CAM was shown to interact with contactin (Brümmendorf et al., 1993) and to initiate second-messenger cascade via its cytoplasmic domain (Doherty and Walsh, 1994). The best-characterized GPI-linked signaling protein is the ciliary neurotrophic factor (CNTF) receptor. Following ligand binding, the CNTF receptor interacts with the signal transducer gp130. The gp130 protein, which is shared by several lymphokines and cytokines, such as interleukin-6, leukemia inhibitory factor, and oncostatin, undergoes dimerization followed by recruitment of the cytoplasmic janus kinase (JAK) protein tyrosine kinases. Stimulation of the JAK kinases leads to activation of both the Ras/MAP kinase and the STAT signaling pathways, which relay signals from the cell surface to the nucleus. It is possible that a contactin-associated protein such as p180 has a function similar to the function of gp130.

The binding of the CAH domain of RPTPβ to contactin leads to cell adhesion and neurite outgrowth. It was recently reported that contactin can mediate the repulsion of neurons by janusin (Pesheva et al., 1993). This effect was proposed to occur in a stepwise manner: first an adhesion step, followed by a signal transduced to the cells, leading to retraction. It appears, therefore, that in response to different stimuli the same molecule can transmit opposite signals, depending on the context or milieu. Whatever the mechanism, the results presented here demonstrate that an RPTP serves as a functional ligand for a GPI-anchored cell adhesion molecule.

We do not know yet whether contactin can serve as a functional ligand for RPTPβ. Modulation of phosphatase activity by neuronal contactin may result in signaling to glial cells. If this does occur, this kind of bidirectional flow of information should allow the interacting cells to respond quickly to local environmental changes during development. Two other RPTPs, RPTPμ and RPTPκ, were shown to mediate cell–cell interaction in a homophilic manner (Brady-Kalany et al., 1993; Gebbink et al., 1993; Sap et al., 1994). However, changes in catalytic activity as a result of these interactions could not be detected. These phosphatases are joining a growing family of proteins involved in cellular recognition that contains intrinsic enzymatic activities, including kinases (Dtrk; Pulido et al., 1992), the β subunit of Na^+, K^+-ATPase (adhesion molecule on glia, or AMOG; Gloor et al., 1990), and the β subunit of prolyl 4-hydroxylase (cognin; Rao and Hausman, 1993).

In summary, we have demonstrated that RPTPβ is a functional ligand for the GPI-anchored cell recognition molecule contactin. The interactions between these two proteins are mediated by the CAH domain of the phosphatase. In addition, the FNIII repeat is required for interaction with glial cells, demonstrating that the multidomain structure of RPTPβ enables interactions with different proteins, which raises the possibility that other potential ligands may modulate these interactions.

Experimental Procedures

Cell Culture

SF763T and SF767T human astrocytoma cell lines were grown in athymic nu/nu mice to create a tumor-derived cell line. The parental lines (SF763 and SF767) were provided by Dr. M. E. Bernes (The Barrow Neurological Institute, Phoenix, AZ). All other cell line were obtained from the American Type Culture Collection (Rockville, MD). Cell cultures of rat sensory neurons were established as described (Hawrot and Patterson, 1979). Neurite outgrowth assays using IMR32 cells and primary tectal neurons were performed as described previously (Grumet et al., 1993).

Generation and Production of Fc Fusion Proteins

Different subdomains of the RPTPβ extracellular region were amplified by polymerase chain reaction (PCR) and cloned into a unique BamHI site upstream of the hinge region of human IgG1–Fc. The βC fusion proteins contained amino acids 1–313, and βCF contained amino acids 1–415 from RPTPβ (Levy et al., 1993). βF–Fc construct was generated by amplification of nucleotides 901–1242 and cloning into pCNγ1 between the IgG gene and a sequence encoding a signal peptide derived from the TGFβ gene (Plowman et al., 1992). The contactin Fc (Hcon–Fc) fusion protein contained amino acids 1–1020 of human contactin fused to the IgG region. Fusion proteins were transiently expressed in COS7 cells or in stably transfected 293 cells and purified on a protein A–Sepharose column (Pharmacia). Immunoblots to detect their expression were done essentially as described (Peles et al., 1992) with anti–human Fc antibodies.

Expression Cloning in COS7 Cells

cDNA was synthesized from GH3–poly(A) mRNA by using Superscript II (GIBCO BRL). cDNAs larger than 2 kb were ligated into a EcoRI- and HindIII-digested pCMP1 plasmid vector, a derivative of the pCMV-1 vector (Lammers et al., 1993). Plasmid DNA (10 μg) was transfected into COS7 cells with lipofectamine (GIBCO BRL). After 72 hr, the cells were incubated with medium containing βCF. Unbound fusion proteins were washed away, and the cells were fixed with 4% paraformaldehyde in PBS. Immunostaining was performed with the ABC staining system (Vector Laboratories), with biotinylated anti–human IgG antibodies followed by streptavidin–alkaline phosphatase and BCIP–NBT as a substrate. One positive pool (number 54) was subdivided and rescreened until a single clone (F8) was isolated.

Construction of RPTPβ–EGF Receptor Chimeras

The region encoding the extracellular domain of the short form of RPTPβ (βCFS, amino acids 1–768) and the region encoding CAH and the FNIII domains (βCF, amino acids 1–418) or the CAH domain alone (βC, amino acids 1–297) were amplified by PCR and attached to the human EGF receptor (EK) at residue 634. The junction of the two molecules was confirmed by nucleotide sequence analysis. The chimeras were subcloned into a NotI site in pCMP1.

Binding Experiments with Fc Fusion Proteins

Confluent monolayers of cells were incubated for 1 hr with conditioned medium containing 0.25–0.50 μg/ml Fc fusion protein. Cells were washed three times with binding medium (0.1% bovine serum albumin, 0.2% nonfat dry milk in DMEM–F12), and bound proteins were detected by additional incubation with ^{125}I–protein A (Amersham). In some experiments, the binding of antibodies against RPTPβ was determined with ^{125}I-labeled anti-mouse IgG (Amersham).

Chemical Covalent Cross-Linking Experiments

Cells were incubated for 1 hr with medium containing the different Fc fusion proteins. Following three washes, the cells were incubated for an additional 30 min with PBS–Ca^{2+} containing 1 mM DTSSP (Pierce, Rockford, IL). Cell lysates were made in SBN lysis buffer (Peles et al., 1991) and incubated with protein A–Sepharose for 2 hr at 4°C. The beads were washed three times with HNTG buffer (Peles et al., 1991), and the bound proteins were eluted by boiling in 1% SDS–PBS. The eluted proteins were diluted with HNTG and subjected to immunoprecipitation with agarose-coupled anti–human IgG antibodies (Sigma).

Protein Purification and Sequencing

Membrane pellet prepared from 5 × 10^8 GH3 cells was resuspended in SML solubilization buffer (2% sodium monolaurate, 2 mM $MgCl_2$, 2 mM PMSF in PBS), diluted 10-fold with PBS containing 2 mM $MgCl_2$, and loaded on a column of βCF–Fc. Bound proteins were separated on 7.5% gel and electroblotted to ProBlott membrane (Applied Biosystems, Incorporated). Microsequencing was performed with an Applied Biosystems model 494 sequencer using standard reagents and programs from the manufacturer. To obtain internal peptide sequence, the blotted band was reduced with 10 mM dithiothreitol and digested for 16 hr with trypsin, and peptides were separated on a 1 mm × 200 mm Reliasil C-18 reverse-phase column on a Michrom UMA high pressure liquid chromatograph (HPLC). Purified peptides were sequenced as described above.

Treatment with PI-PLC

Cells were metabolically labeled with 100 μCi/ml [^{35}S]methionine (New England Nuclear, Boston, MA) and incubated with 250 mU of PI-PLC (Boehringer Mannheim) for 50 min at 37°C. The supernatant was collected and cleared by centrifugation, and membranes were prepared from the cells and solubilized in SML buffer. βCF–Fc bound to protein A–Sepharose beads was added to the supernatant and the membrane fractions for 2 hr at 4°C. The beads were washed twice with 0.15% sodium monolaurate in PBS and once in PBS prior to the addition of SDS sample buffer.

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