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Receptor Tyrosine Phosphatase β Is Expressed in the Form of Proteoglycan and Binds to the Extracellular Matrix Protein Tenascin*

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The extracellular domain of receptor type protein tyrosine phosphatase β (RPTPβ) exhibits striking sequence similarity with a soluble, rat brain chondroitin sulfate proteoglycan (3F8 PG). Immunoprecipitation experiments of cells transfected with RPTPβ expression vector and metabolically labeled with [^{35}S]sulfate and [^{35}S]methionine indicate that the transmembrane form of RPTPβ is indeed a chondroitin sulfate proteoglycan. The 3F8 PG is therefore a variant form composed of the entire extracellular domain of RPTPβ probably generated by alternative RNA splicing. Previous immunohistochemical studies indicated that both RPTPβ and the extracellular matrix protein tenascin are localized in similar regions of the central nervous system. We have performed co-aggregation assays with red and green Covaspheres coated with tenascin and 3F8 PG, respectively, showing that the extracellular domain of RPTPβ (3F8 PG) binds specifically to tenascin. The interaction between a receptor tyrosine phosphatase and an extracellular matrix protein may have a role in development of the mammalian central nervous system.

Cellular tyrosine phosphorylation plays a crucial role in the control of cell growth and differentiation of eukaryotes. Tyrosine phosphorylation is regulated by the balanced action of protein tyrosine kinases and protein tyrosine phosphatases (PTP).[1] One class of PTPs encompasses membrane proteins composed of an extracellular domain, a single transmembrane domain, and a cytoplasmic region with one or usually two tandem catalytic tyrosine phosphatase domains (reviewed by Fischer *et al.* (1991) and Saito and Streuli (1991)). RPTPβ (also known as HPTPζ), together with RPTPγ, define a subfamily of membrane-bound protein tyrosine phosphatases (Kaplan *et al.*, 1990; Krueger and Saito, 1992; Barnea *et al.*, 1993; Levy *et al.*, 1993). This subfamily is characterized by extracellular portions consisting of an amino-terminal carbonic anhydrase-like domain followed by a fibronectin type III repeat and by a cysteine-free domain of variable length (Barnea *et al.*, 1993).

RPTPβ is expressed predominantly in the developing central nervous system, and its spatial and temporal pattern of expression suggests that it plays a role in morphogenesis and plasticity of the nervous system. High levels of RPTPβ are expressed in a variety of cells of neuroectodermal origin. For example, RPTPβ immunoreactivity is detected in the processes of radial glia, which act as guides during neuronal migration and axonal elongation. High levels of RPTPβ immunoreactivity are also seen in nerve fiber tracts throughout the central and peripheral nervous system during periods of axonal growth. In the adult, high levels of RPTPβ are seen in regions of the brain where there is continued neurogenesis and neurite outgrowth, such as the subependymal layer of the lateral ventricle and the olfactory nerve layer of the olfactory bulb (Canoll *et al.*, 1993).

Proteoglycans are proteins that contain one or more glycosaminoglycan (GAG) chains (reviewed by Kjellén and Lindahl (1991)). GAGs are classified based on their disaccharide structures: heparan sulfate and heparin, chondroitin sulfate and dermatan sulfate, keratan sulfate and hyaluronic acid. The sulfated GAGs occur in tissues in the form of proteoglycans, having a strong negative charge. Proteoglycans are involved in a variety of molecular interactions, which may be mediated by the GAG component or by the core protein. Proteoglycans mediate cell-cell and cell-matrix interactions and bind growth factors, such as fibroblast growth factor (reviewed by Ruoslahti and Yamaguchi (1991)). A number of proteoglycans are expressed in nervous tissue (reviewed by Margolis and Margolis (1993)). Chondroitin sulfate proteoglycans have been implicated in neural crest cell migration (Perris and Johansson, 1990) and have been detected in several glial cell structures that appear to act as barriers to axonal growth, such as the roof plate of the spinal cord and optic tectum (Snow *et al.*, 1990). Interestingly, high levels of RPTPβ are also found in these barrier structures, consistent with the hypothesis that RPTPβ may be involved in guidance mechanisms (Canoll *et al.*, 1993). It has also been shown that certain rat brain proteoglycans are able to bind directly to neurons and to inhibit the self-aggregation of beads coated with the cell adhesion molecules Ng-CAM and N-CAM, suggesting that Ng-CAM and N-CAM may act as heterophilic ligands for these proteoglycans (Grumet *et al.*, 1993).

Tenascin is a large oligomeric extracellular matrix glycoprotein that is widely expressed during embryonic development and is also known as cytotactin and hexabrachion (reviewed by Erickson and Bourdon (1989) and by Chiquet *et al.* (1991)). In the nervous system tenascin is associated with migration pathways of neural crest cells and with outgrowing peripheral nerves (reviewed by Chiquet *et al.* (1991)). It is present in high concentration in areas such as the molecular layer of the cerebellum (Grumet *et al.*, 1985) and marks boundaries between developing functional units, such as the walls of the barrel fields of somatosensory cortex (Steindler *et al.*, 1989; Crossin *et al.*, 1989). Glial tenascin may modulate axon fasciculation and

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[1] The abbreviations used are: PTP, protein tyrosine phosphatase; RPTPβ, receptor type protein tyrosine phosphatase β; PG, proteoglycan; GAG, glycosaminoglycan; BSA, bovine serum albumin; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; kb, kilobase(s); N-CAM, neural cell adhesion molecule; Ng-CAM, neuron-glia cell adhesion molecule.

help to confine neuronal pathways while allowing plasticity during development and regeneration. This matrix protein has multiple domains that bind to cells and mediate cell adhesion as well as domains that prevent cell spreading and cell migration in model systems (Prieto *et al.*, 1992; Lochter *et al.*, 1991).

In this report, we show that RPTPβ is expressed in the form of chondroitin sulfate proteoglycan in transfected cells. Because of the similar cellular localization of RPTPβ and tenascin in radial glial cells of the central nervous system, we have examined the interaction between these two proteins utilizing a coaggregation assay. These experiments indicate that tenascin binds specifically to the extracellular domain of RPTPβ. The interaction between an extracellular matrix protein and a receptor-linked tyrosine phosphatase may play a role in the development of the central nervous system.

EXPERIMENTAL PROCEDURES

Proteins—Purification and characterization of the proteoglycans 3F8 and aggrecan have been described (Rauch *et al.*, 1991; Faltz *et al.*, 1979). Ng-CAM (Grumet and Edelman, 1988) was purified from 14-day embryonic chicken brains. Tenascin (Telios) and human plasma BSA (ICN Biomedical, Lisle, IL) were obtained commercially. Monoclonal antibody 10F6 against chicken Ng-CAM (Grumet and Edelman, 1984) and the 3F8 monoclonal antibody (Rauch *et al.*, 1991) have been described. The 4H7 monoclonal antibody to the 3F8 proteoglycan was provided by R. K. Margolis. Rabbit antibodies against chicken cytotactin/tenascin were prepared (Grumet *et al.*, 1985). Igs and Fab′ fragments were prepared as described (Brackenbury *et al.*, 1977).

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from adult mouse brain and samples containing 20 μg of total RNA were resolved and probed by standard procedures (Levy *et al.*, 1993).

Transfection, Labeling, and Immunoprecipitation—Subconfluent 10-cm diameter dish cultures were transfected by the modified calcium phosphate technique (Chen and Okayama, 1987). At 48–72 h after transfection, the cells were metabolically labeled either for 5 h in methionine-free medium containing [^{35}S]methionine (ICN) at 100 μCi/ml or for 20 h in sulfate-free medium (NEX-041H medium) containing [^{35}S]sulfate (ICN) at 200 μCi/ml. Cell lysis and immunoprecipitation were done as previously described (Levy *et al.*, 1993).

Chondroitinase ABC Treatment—Immunoprecipitates were washed twice more with chondroitinase buffer (100 mM Tris, 30 mM sodium acetate, HCl to adjust the pH to 8.3 at 25 °C), and the beads were resuspended in 100 μl of chondroitinase buffer. Protease-free chondroitinase ABC (Seikagaku America, Inc.) was added to a final concentration of 0.5 milliunit/μl and incubated at 37 °C for 1 h with agitation.

Covasphere Aggregation—Proteins (50 μg) were covalently coupled to 200 μl of 0.5-μm Covaspheres (Duke Scientific Corp., Palo Alto, CA), washed twice in PBS containing 1 mg/ml BSA/10 mM NaN_3, and resuspended in 200 μl of buffer as described (Grumet and Edelman, 1988). Coaggregation of different colored Covaspheres was performed in a total volume of 50 μl of PBS, 0.1 mg/ml BSA using mixtures of 5 μl of red-fluorescing tenascin-coated Covaspheres and 2 μl of green-fluorescing Covaspheres derivatized with 3F8 PG, aggrecan, Ng-CAM, or BSA. When antibodies were tested, Fab′ fragments were preincubated with the Covaspheres on ice for 15 min. The mixtures were sonicated for 10–20 s and visualized under a microscope to verify that they were not aggregated. The samples were then incubated without agitation at room temperature (25–28 °C). After 2 h they were visualized under a Nikon fluorescence microscope, and green- and red-fluorescing Covaspheres were photographed individually with filters that discriminate between the dyes. Coaggregation was measured using a fluorescence-activated cell sorter (Becton Dickinson FACScan) as described previously (Kuhn *et al.*, 1991). The system was calibrated using green- and red-fluorescing Covaspheres that do not interact with themselves or with other Covaspheres. With such samples, thresholds were established to reject aggregates of Covaspheres of a single color, and mixed aggregates were counted only when they contained at least three red- and three green-fluorescing Covaspheres.

RESULTS AND DISCUSSION

A PBS-soluble chondroitin sulfate proteoglycan of rat brain termed phosphacan, isolated with the 3F8 monoclonal antibody, has been biochemically characterized (Rauch *et al.*, 1991) and cloned (Maurel *et al.*, 1994). The NH_2-terminal amino acid

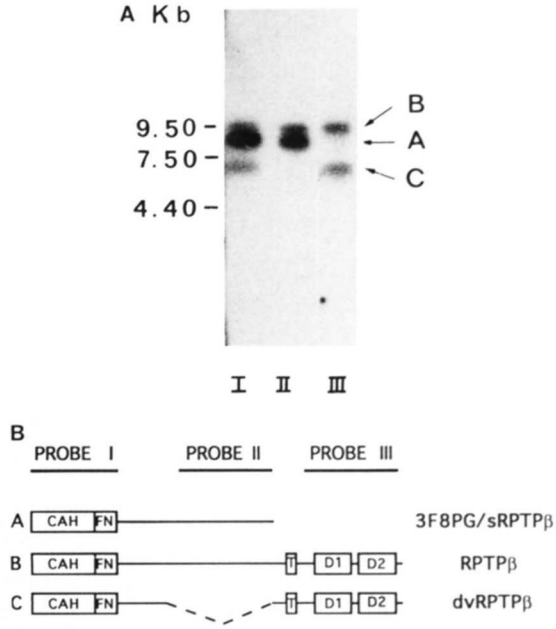

FIG. 1. **RPTPβ is expressed in three different forms.** Total RNA isolated from adult mouse brain was separated on a formaldehyde-containing gel and analyzed (*panel A*) with probes corresponding to three different parts of RPTPβ that were amplified by PCR (*panel B*). Band A was not detected by probe III, which is derived from the cytoplasmic portion of RPTPβ, and therefore corresponds to the secreted form 3F8/sRPTPβ. Band B was detected by all three probes and therefore corresponds to the long form of RPTPβ. Band C was not detected by probe II, which is derived from the region deleted in the variant cDNA clone, and therefore corresponds to the deletion variant dvRPTPβ. *Panel B* shows a schematic diagram of the three forms of RPTPβ. All three forms contain the carbonic anhydrase-like domain (*CAH*) and the fibronectin type III repeat (*FN*). However, only two forms contain the transmembrane domain (*T*) and the two phosphatase domains (*D1* and *D2*). The sequence that is deleted in the deletion variant is indicated by the *dotted line*. The positions of the three probes that were generated by PCR and used in Northern analysis are indicated with *Roman numerals*. The *letters A, B,* and *C* correspond to the specific bands (9.5, 8.5, and 6.4 kb) that were detected in the Northern blot.

sequence of 3F8 PG contains a domain with homology to the enzyme carbonic anhydrase. We have previously shown that carbonic anhydrase-like domains exist in the extracellular domains of two receptor type tyrosine phosphatases RPTPβ (or HPTPζ) and RPTPγ (Levy *et al.*, 1993; Krueger and Saito, 1992; Barnea *et al.*, 1993). Comparison of the amino acid sequences of these regions revealed a striking sequence similarity between RPTPβ and the 3F8 PG, which suggests that the proteoglycan represents the rat homologue of RPTPβ (Levy *et al.*, 1993; Maurel *et al.*, 1994; Barnea *et al.*, 1994). Complementary DNA cloning and Northern analysis provided evidence for at least two forms of human RPTPβ probably generated by alternative splicing (Levy *et al.*, 1993). Molecular analysis of the rat extracellular 3F8 PG suggests that it is encoded by an additional mRNA splicing variant (Maurel *et al.*, 1994; Barnea *et al.*, 1994). To characterize the relationship between the three forms of the mRNAs in a single species, we prepared cDNA probes from three non-overlapping regions of the full-length sequence of RPTPβ and performed Northern analysis. The 9.5- and 6.4-kb transcripts encode the large and small transmembrane forms of RPTPβ, respectively, whereas the 8.5-kb transcript encodes a secreted form of the extracellular domain of RPTPβ (Fig. 1). Analysis of the complete sequence of 3F8 PG cDNA (encoding 1616 amino acids) confirmed that it represents the rat homologue of RPTPβ, corresponding to its entire extracellular domain (Maurel *et al.*, 1994; Levy *et al.*, 1993).

We therefore examined the possibility that human RPTPβ also appears in a form of a proteoglycan. Human 293 cells were

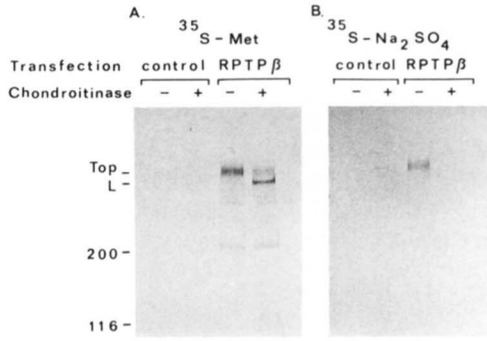

FIG. 2. **RPTPβ is a chondroitin sulfate proteoglycan.** Human fibroblast cells (293) were transfected with either full-length RPTPβ in a cytomegalovirus based expression vector or vector alone as a control. The cells were then labeled with either [^{35}S]methionine (*panel A*) or [^{35}S]sulfate (*panel B*), lysed, and immunoprecipitated with anti-RPTPβ antibodies. The immunocomplexes were divided into two samples, one of which was treated with the enzyme chondroitinase ABC, and were resolved on 5% acrylamide gels. Molecular size markers (in kDa) are noted on the *left*, *L* denotes the position of the 440-kDa form of laminin, and the top of the gel is indicated. Note the appearance of a new band upon treatment of [^{35}S]methionine-labeled cells with chondroitinase ABC (*panel A*) and the disappearance of the band upon treatment of the [^{35}S]sulfate-labeled cells with the enzyme.

transfected with an expression vector that encodes for the full-length RPTPβ (large form) or with vector alone. The cells were then labeled with either [^{35}S]methionine or [^{35}S]sulfate, lysed, and immunoprecipitated with anti-RPTPβ antibodies. The immunocomplexes were divided into two samples. One sample was treated with chondroitinase ABC, an enzyme that specifically depolymerizes chondroitin sulfate to disaccharides. RPTPβ migrated on SDS-polyacrylamide gels as a broad band that poorly entered the gel, a characteristic of proteoglycans; this band did not appear in control-transfected cells (Fig. 2*A*). Upon treatment with chondroitinase ABC a new band appeared which migrated with a more uniform mobility. We performed a similar experiment using transfected cells labeled with [^{35}S]sulfate, which labels the sulfated glycosaminoglycan chains (Fig. 2*B*). While RPTPβ from transfected cells was labeled with [^{35}S]sulfate, no band was present in immunoprecipitates from control cells transfected with vector alone. Treatment with chondroitinase ABC resulted in the disappearance of the sulfate-labeled RPTPβ. However, the RPTPβ polypeptide core was still present, as demonstrated by chondroitinase treatment of immunoprecipitates from [^{35}S]methionine-labeled cells. This result indicates that the [^{35}S]sulfate was incorporated into RPTPβ in the form of chondroitin sulfate. Hence, RPTPβ is expressed in the form of proteoglycan. In addition, RPTPβ is a glycoprotein that contains *N*-linked carbohydrates (Levy *et al.*, 1993). Indeed, the band that appeared upon chondroitinase ABC treatment was also found to migrate faster on SDS-polyacrylamide gels in experiments in which tunicamycin was added to the transfected cells (data not shown). This observation indicates that the same core protein contains both chondroitin sulfate and *N*-linked carbohydrates.

RPTPβ is the first tyrosine phosphatase shown to be a proteoglycan and is the first cell surface proteoglycan that contains a cytoplasmic domain with a known catalytic activity. It is possible, however, that other receptor-linked protein tyrosine phosphatases may also be expressed in the form of proteoglycans. Interestingly, other PTPs such as CD45, RPTPα, and RPTPγ contain in their extracellular domains clusters of amino acid sequences that are rich in serine and threonine residues. It had been suggested that these proteins contain *O*-linked, as well as *N*-linked carbohydrates (Saito and Streuli, 1991).

Since the overall pattern of expression of RPTPβ in the developing rat cerebellum (Canoll *et al.*, 1993) resembles that of

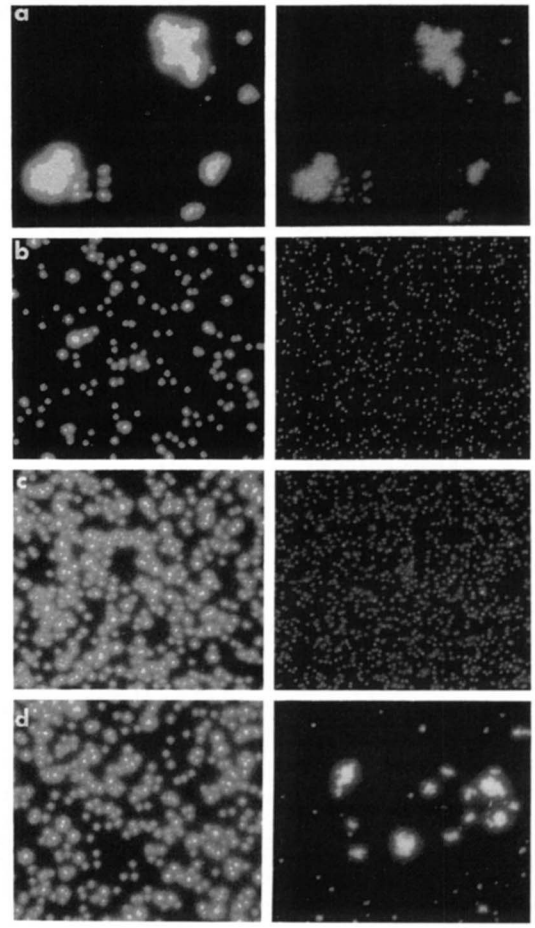

FIG. 3. **Coaggregation of tenascin-coated Covaspheres with 3F8 proteoglycan-coated Covaspheres.** Red-fluorescing Covaspheres coated with tenascin (*left panels*) were mixed with green-fluorescing Covaspheres (*right panels*) coated with 3F8 proteoglycan (*a* and *b*), aggrecan (*c*), and Ng-CAM (*d*). The experiment shown in *panel b* is identical to that in *panel a*, except that it was performed in the presence of Fab′ fragments of 3F8 monoclonal antibody. Control antibodies did not inhibit coaggregation as shown, for example, in Table I. Identical fields were visualized specifically for red-fluorescing tenascin-coated Covaspheres and green-fluorescing Covaspheres and were photographed under a fluorescence microscope as described under "Experimental Procedures." Note that in the matched pairs of micrographs, the patterns of aggregates of green- and red-fluorescing particles are nearly identical only in *panel a*.

the matrix protein tenascin (Prieto *et al.*, 1990), we investigated whether these two proteins are able to interact with each other. We used a coaggregation assay in which red-fluorescing tenascin-coated Covaspheres were incubated with green-fluorescing Covaspheres coated with 3F8 PG, aggrecan (a chondroitin sulfate proteoglycan isolated from rat cartilage), or Ng-CAM. After 2 h of incubation at room temperature without agitation, coaggregation was visualized under the fluorescence microscope and measured using a fluorescence-activated cell sorter. Tenascin-coated Covaspheres coaggregated with 3F8 PG-coated Covaspheres, forming large aggregates (Fig. 3*a*). The coaggregation was disrupted by a monoclonal antibody against 3F8 PG (Fig. 3*b*). Tenascin-coated Covaspheres did not form aggregates with Covaspheres coated with the proteoglycan aggrecan (Fig. 3*c*) or with Ng-CAM (Fig. 3*d*), which binds in a homophilic manner and forms self-aggregates (Grumet and Edelman, 1988).

To quantitate the coaggregation (Table I), mixed aggregates were counted using fluorescence-activated cell sorter analysis only when they contained at least three fluorescing Covaspheres of each color. The aggregation of tenascin-coated Covaspheres with 3F8 PG-coated ones was resistant to chondroitinase treat-

TABLE I
Quantitative analysis of coaggregation of tenascin-coated Covaspheres with Covaspheres coated with 3F8 PG

Covaspheres were mixed on ice, dissociated by sonication, and incubated for 2 h. at room temperature. Aliquots (10 μl) were diluted into 1 ml of PBS and subjected to analysis by FACS as described under "Experimental Procedures." Fab' fragments of antibodies and other proteins in solution were used at 40 μg/ml except for polyclonal rabbit antibodies (non-immune and anti-tenascin), which were used at 400 μg/ml; several antibodies that do not recognize either tenascin or 3F8 PG did not inhibit coaggregation of these proteins on Covaspheres. mAb, monoclonal antibody.

Protein on red Covaspheres	Protein on green Covaspheres	Protein in solutoin	No. of mixed aggregates
Tenascin	3F8 PG		5,169 ± 1,318
Tenascin	3F8 PG + chondroitinase		6,339 ± 851
Tenascin	3F8 PG	Anti-tenascin	152 ± 149
Tenascin	3F8 PG	mAb 4H7	68 ± 5
Tenascin	3F8 PG	mAb 10F6	4,990 ± 1,294
Tenascin	3F8 PG	3F8 PG	129 ± 63
Tenascin	3F8 PG	Aggrecan	4,936 ± 1,548
Tenascin	Ng-CAM		115 ± 83
Tenascin	BSA		189 ± 141
Tenascin	Aggrecan		208 ± 115

ment, suggesting that the chondroitin sulfate was not necessary for binding. The specificity of this interaction was confirmed by the observations that the aggregation was disrupted by antibodies against either tenascin or 3F8 PG (monoclonal antibody 4H7) and was resistant to monoclonal antibodies (10F6) and polyclonal antibodies (data not shown) that do not bind to either tenascin or 3F8 PG. Tenascin-coated Covaspheres did not interact with other proteins on Covaspheres. Addition of 3F8 PG to the mixture inhibited the coaggregation, probably by competition, whereas addition of aggrecan had no effect.

It has been reported that mice with a disrupted tenascin gene do not exhibit any apparent phenotype (Saga *et al.*, 1992). This may be due to functional redundancy, since there are other known members of the tenascin family such as restrictin (Nörenberg *et al.*, 1992). It is thought that tenascin plays a role in morphogenesis and plasticity of the nervous system. The interaction between tenascin and RPTPβ may be important for these biological functions. Based on the structure of tenascin and RPTPβ and their patterns of expression, there are several possible mechanisms by which the interaction between these two proteins may modulate their function. The binding of tenascin to RPTPβ may activate, inactivate, or change the subcellular distribution of RPTPβ. The interaction of tenascin with RPTPβ may also relocalize a constitutively active RPTPβ either away from or toward its biological substrate, and thereby modulate the phosphorylation state of the substrate in local regions of contact with tenascin. Yet another possible role for the interaction between RPTPβ and tenascin is to target RPTPβ to another protein that may modulate the activity of RPTPβ. Tenascin has been shown to inhibit the proliferation of fibroblasts in response to growth factors or the tumor promoter phorbol 12-myristate 13-acetate (Crossin, 1991). However, the molecular mechanisms underlying this inhibition are not understood. It has been demonstrated that the adhesion molecules N-CAM and Ng-CAM also bind to 3F8 PG (Grumet *et al.*, 1993; Milev *et al.*, 1993). RPTPβ is, therefore, able to interact not only with a cell matrix protein but also with cell adhesion molecules. In this regard, it is interesting that recent studies demonstrated that members of another subfamily of receptor type tyrosine phosphatases, termed RPTPμ and RPTPκ, undergo homophilic interactions leading to cell aggregation. So far, it has been impossible to detect any change in tyrosine phosphorylation following cell aggregation mediated by these two receptor-linked phosphatases (Gébbink *et al.*, 1993; Brady-Kalnay *et al.*, 1993; Sap *et al.*, 1993).

In order to elucidate the biological role of the interaction between tenascin and RPTPβ, it will be necessary to establish stable cell lines that overexpress the full-length phosphatase and to identify the specific *in vivo* substrates of RPTPβ. Moreover, since the changes in activity may be local, individual cell recording assays may be required. The identification of RPTPβ as a proteoglycan that binds in a heterophilic manner to tenascin and other cell surface proteins may provide some of the tools to address the issue of modulation of tyrosine phosphatase activity by extracellular and cell surface proteins.

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