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Characterization and Developmental Regulation of Proteoglycan-Type Protein Tyrosine Phosphatase ζ/RPTPβ Isoforms[1]

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Protein tyrosine phosphatase ζ (PTPζ/RPTPβ) is a receptor-like protein tyrosine phosphatase specifically expressed in the brain. Alternative splicing produces three isoforms of this molecule: PTPζ-A, the full-length form of PTPζ; PTPζ-B, the short form of PTPζ; and PTPζ-S, an extracellular variant. Here, we identified all these isoforms, including PTPζ-B, as chondroitin sulfate proteoglycans, and characterized their carbohydrate modification and expression profiles in the rat brain. The level of PTPζ-A expression was maintained during the prenatal period and decreased rapidly after birth. PTPζ-S was expressed in a similar manner, although the postnatal decrease was gradual. In contrast, relatively constant amounts of PTPζ-B were observed from embryonic day 13 (E13) through adulthood. PTPζ-A and -S were constantly expressed only as proteoglycans during development, but a substantial amount of PTPζ-B was detected in a non-proteoglycan form at E13-15. Moreover, PTPζ-B did not contain Le^{X}, HNK-1 carbohydrate, or keratan sulfate, although PTPζ-A and -S were generally modified with these carbohydrates. L cells transfected with PTPζ-A and -B cDNAs expressed these proteins as enzymatically active chondroitin sulfate proteoglycans. The PTPζ-A and -B in L cells showed essentially similar localizations in cell cortical structures on immunofluorescence microscopy, although immature or processed forms of PTPζ-A were accumulated additively in intracellular patchy structures. These results show that the three isoforms of PTPζ are differentially regulated during development, and that the extracellular deleted region in PTPζ-B is important for determination of carbohydrate modification.

Key words: brain development, carbohydrate modification, chondroitin sulfate proteoglycan, protein tyrosine phosphatase, subcellular localization.

Cellular proliferation, differentiation and adhesion are regulated through protein tyrosine phosphorylation mediated by protein tyrosine kinases and protein tyrosine phosphatases (PTPs) (*1-4*). The protein tyrosine phosphatases are a diverse family of cytoplasmic and transmembrane receptor-like enzymes. Receptor-like PTPs consist of an extracellular region with various domain structures, a transmembrane segment, and one or two intracellular phosphatase domains. The tyrosine phosphatase activities of these molecules are thought to be regulated by specific ligands which bind to their extracellular segments (*1-4*).

PTPζ/RPTPβ is a proteoglycan-type receptor-like PTP specifically expressed in the brain (*5-9*). The extracellular region of PTPζ consists of an N-terminal carbonic anhydrase-like domain, a fibronectin type III domain, and a large cysteine-free, serine-glycine-rich region (*5, 6*). An extracellular variant, known as 6B4 proteoglycan or phosphacan, occurs as a major soluble chondroitin sulfate proteoglycan in the brain (*7, 8*). It is well established now that three splice variants of this molecule are expressed in the brain: (1) the full-length form of PTPζ, (2) the short form of PTPζ, in which the greater part of the cysteine-free, serine-glycine-rich region is deleted, and (3) 6B4 proteoglycan/phosphacan (*5-9*). In this paper, we refer to these variants as PTPζ-A, PTPζ-B, and PTPζ-S, respectively, and as PTPζ collectively. Among them, PTPζ-A and -S have been identified as chondroitin sulfate proteoglycans (*7, 10, 11*), but almost nothing is known about PTPζ-B.

The expression of PTPζ is spatiotemporally regulated in the brain, suggesting its involvement in neuronal cell migration, differentiation, and specific circuit formation (*12-20*). PTPζ binds various cell adhesion molecules and extracellular matrix molecules. The carbonic anhydrase-like domain of PTPζ binds F3/contactin, and it has been suggested that PTPζ on glial cells acts as a ligand for F3/contactin expressed on neurons (*21, 22*). Ng-CAM/L1, N-

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Abbreviations: CMF-HBSS, Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution; HB-GAM, heparin-binding growth-associated molecule; Le^{X}, Lewis X; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; PTP, protein tyrosine phosphatase; RPTP, receptor-like protein tyrosine phosphatase.

CAM, and tenascin have been reported to bind to PTPζ-S at least in part through *N*-linked oligosaccharides (*10, 23-25*). PTPζ-S also binds to TAG-1/axonin-1 with high affinity, and chondroitinase ABC digestion of PTPζ-S decreased the binding by ~70% (*26*). We demonstrated that pleiotrophin/heparin-binding growth-associated molecule (HB-GAM) binds to PTPζ-S (*27*). The binding affinity of PTPζ-S as to pleiotrophin is regulated by chondroitin sulfate chains which constitute part of the binding site. Furthermore, we recently reported that keratan sulfate modification of PTPζ-A and -S is developmentally regulated in the chick brain, especially at the mes-metencephalic boundary (*28*). These results suggest that carbohydrate modifications of PTPζ play an important role in the regulation of ligand binding. However, little is known about the differences in the carbohydrate modification of the PTPζ isoforms.

In this study, we identified PTPζ-B from rat brain as a chondroitin sulfate proteoglycan with 220 kDa core protein. In contrast to PTPζ-A and -S, which were constantly expressed as chondroitin sulfate proteoglycans, chondroitin sulfate modification of PTPζ-B was developmentally regulated. In the early prenatal period, a substantial amount of non-proteoglycan-type PTPζ-B was detected, but after birth most of this isoform was expressed as chondroitin sulfate proteoglycan. Furthermore, we found that PTPζ-B was not modified with Le^X, HNK-1 carbohydrate, or keratan sulfate, all of which were attached to PTPζ-A and -S. It was also found that the cDNA-derived PTPζ-A and -B proteins expressed in L cells exhibited localization in cell cortical structures such as ruffling membranes.

MATERIALS AND METHODS

Materials—DEAE-Toyopearl 650M was purchased from Tosoh. Protein G Sepharose 4FF was obtained from Pharmacia Biotech. Protease-free chondroitinase ABC, anti-Le^X monoclonal antibody, and anti-keratan sulfate monoclonal antibody 5-D-4 were purchased from Seikagaku. HNK-1 monoclonal antibody was obtained from Serotec. Anti-6B4 proteoglycan antiserum and antiserum 31-5 were described previously (*7*). Anti-6B4 proteoglycan antibody (IgG fraction from anti-6B4 proteoglycan antiserum) was prepared as described in Ref. *29*. Dulbecco's modified Eagle's medium, F12 medium, and B-27 supplement were purchased from Life Technologies. G418, soybean trypsin inhibitor, and poly-L-lysine ($M_r > 300{,}000$) were obtained from Sigma. pcDNAI was obtained from Invitrogen. DNAase I was purchased from Boehringer Mannheim. Anti-RPTPβ was obtained from Transduction Laboratories. Biotinylated anti-mouse Ig, biotinylated anti-rabbit Ig, and Texas Red Avidin D were obtained from Amersham. FITC-conjugated anti-mouse IgG was purchased from Jackson Immunoresearch. Texas Red-conjugated anti-rabbit IgG was obtained from Oreganon Teknika. FITC-conjugated phalloidin was obtained from Molecular Probes.

Partial Purification of Proteoglycan-Type PTPs—Partial purification of proteoglycan-type PTPs was carried out as described previously (*7*). Briefly, 8 g of whole brains from 0- and 20-day-old Sprague-Dawley rats was homogenized in 50 ml of 5 mM EDTA/1 mM dithiothreitol (DTT)/0.1 mM phenylmethylsulphonyl fluoride (PMSF)/10 μM leupeptin/10 μM pepstatin A/1 mM benzamidine/50 mM Tris-HCl, pH 7.4 (buffer A), containing 0.32 M sucrose. The homogenate was centrifuged at 1,000 × *g* for 5 min at 2°C, and the resultant precipitate was washed under the same conditions. The combined supernatants were centrifuged at 105,000 × *g* for 60 min at 2°C to precipitate the postnuclear fraction. Proteins were solubilized in 50 ml of buffer A containing 1% (w/v) CHAPS and 0.1 M NaCl, and insoluble materials were removed by centrifugation at 20,000 × *g* for 60 min at 2°C. The supernatant was applied to a DEAE-Toyopearl column (10 ml) equilibrated with 0.5% CHAPS/1 mM EDTA/1 mM DTT/0.1 mM PMSF/10 μM leupeptin/10 μM pepstatin A/1 mM benzamidine/50 mM Tris-HCl, pH 7.4 (buffer B), containing 0.1 M NaCl. The column was washed with 80 ml of 0.25 M NaCl/buffer B, and then the proteins were eluted with 0.6 M NaCl/buffer B. After adjusting the density to 1.43 g/ml with solid CsCl, the eluate was centrifuged at 77,000 rpm for 36 h at 4°C in a Beckman TLA100.4 rotor. The samples in centrifugation tubes were divided into 10 fractions according to density, and then used for immunoblotting analysis and measurement of PTP enzymatic activity as described previously (*7*).

Preparation of the Brain Extract—Whole brains were homogenized in 5 volumes of buffer A containing 0.32 M sucrose. The homogenates were centrifuged at 1,000 × *g* for 5 min at 2°C, and the resultant supernatants were centrifuged at 105,000 × *g* for 60 min at 2°C. The postnuclear fractions thus obtained were solubilized in 0.2% (w/v) Triton X-100/0.1% (w/v) sodium deoxycholate/1 mM PMSF/10 μM pepstatin A/10 μM leupeptin/1 mM EDTA/20 mM Tris-HCl, pH 7.4/0.15 M NaCl (buffer C). After centrifugation at 20,000 × *g* for 60 min at 2°C, the supernatants were used for immunoprecipitation.

Immunoprecipitation and Immunoblotting—The protein concentration of each sample was first adjusted to 50 μg/ml with buffer C. The samples (400 μl) were preadsorbed with Protein G Sepharose 4FF (40 μl) by rotating the sample tubes for 2 h at 4°C. After centrifugation, the supernatants were mixed with 5 μg of the anti-6B4 proteoglycan antibody (*29*), and then incubated for 3 h at 4°C. Then, 25 μl of Protein G Sepharose 4FF was added to the samples, followed by incubation for 3 h at 4°C. The gel was washed 2 times with buffer C and 2 times with PBS, and then mixed with the same volume of 10 mM EDTA/2 mM PMSF/0.2 mM pepstatin A/60 mM sodium acetate/0.2 M Tris-HCl, pH 7.5. The samples were incubated for 1 h at 37°C in the presence or absence of protease-free chondroitinase ABC (0.4 U/ml).

Samples were treated with SDS-PAGE sample buffer, subjected to 5% SDS-PAGE (*30*), and then processed for immunoblotting as described (*13*). The antibodies were used at the following dilutions: anti-6B4 proteoglycan antiserum, 1:1,000; antiserum 31-5, 1:100; and anti-RPTPβ, 1:100.

Isolation of L Cell Transfectants—Full-length rat PTPζ-A and PTPζ-B cDNAs (7) were subcloned into the mammalian expression vector, pcDNAI, to yield pcDPG-503 and pcDPG-504, respectively, in which constitutive expression is directed from the cytomegalovirus promoter. L cells (5×10^5 cells/60 mm dish) were plated and grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. The cells were transfected by calcium phosphate coprecipitation using 25 μg of either pcDPG-503 or pcDPG-504, together with 2.5 μg of pSTneoB which encodes the neomysin resistance gene. The cells were glycerol shocked for 90 s after 8 h incubation and then the medium was changed to one containing 400 μg/ml G418 after 24 h. Stable G418-resistant clones were isolated after 2-3 weeks, and the expression of PTPζ isoforms was examined by immunofluorescence microscopy using the anti-6B4 proteoglycan.

Analysis of L Cell Transfectants—L cell transfectants producing PTPζ-A or -B were plated (1×10^6 cells per 100 mm dish) and cultured for 12 h. The cell layers were washed with ice-cold PBS, and then extracted with 2 ml of 1% (w/v) CHAPS/1 mM PMSF/0.1 mM pepstatin A/10 μM leupeptin/1 mM EDTA/10 mM Tris-HCl, pH 7.4 (buffer D), for 30 min at 4°C. After centrifugation at 15,000 × g for 15 min, the supernatant was applied to a 200 μl column of DEAE-Toyopearl, which was then washed with 0.2% (w/v) CHAPS/1 mM EDTA/1 mM PMSF/0.1 mM pepstatin A/10 μM leupeptin/10 mM Tris-HCl, pH 7.4 (buffer E), containing 0.25 M NaCl. Proteins were eluted from the column with 0.6 M NaCl/buffer E, and then precipitated with ethanol. The precipitated proteins were treated with chondroitinase ABC and then subjected to immunoblotting as described above.

Assaying of PTP Activity—PTP assaying of L cell transfectants was performed as follows. Cell layers cultured for 12 h were washed with ice-cold PBS, and then extracted with 1 ml of 1% (w/v) CHAPS/1 mM DTT/0.1 mM PMSF/10 μM pepstatin A/10 μM leupeptin/5 mM EDTA/0.15 M NaCl/50 mM Tris-HCl, pH 7.5 (buffer F), for 30 min at 4°C. After centrifugation at 15,000 × g for 15 min, the supernatants were mixed with 5 μg of the anti-6B4 proteoglycan antibody or rabbit IgG, and then incubated for 2 h at 4°C. After mixing with 30 μl of Protein G Sepharose 4FF, the samples were incubated for 1 h at 4°C. The gel was washed 3 times with buffer F and then assayed for PTP activity using [^{32}P]phosphotyrosine-labeled Raytide as a substrate as described (*7*).

Primary Culture of Dissociated Cerebral Neurons—Cerebra were dissected out from E17 Sprague-Dawley rats and the meninges were removed. The tissues were incubated first in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS) containing 0.1% (w/v) trypsin for 15 min at 37°C. After three washes with CMF-HBSS, the tissues were triturated in CMF-HBSS containing 0.025% (w/v) DNAase I/0.4 mg/ml soybean trypsin inhibitor/3 mg/ml BSA/12 mM MgCl$_2$ with Pasteur pipettes. The cell suspension was centrifuged at 160 × g for 5 min at 4°C, and then the pelleted cells were washed once with CMF-HBSS. Cells were resuspended in a culture medium comprising a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 medium containing 2% (v/v) B-27 supplement, and then seeded onto poly-L-lysine-coated coverslips as described previously (*13*).

Double Immunofluorescence Staining—L cell transfectants were washed once with PBS and then fixed with 4% (w/v) paraformaldehyde/0.1 M sodium phosphate buffer, pH 7.5, for 20 min. The fixed cells were rinsed three times with PBS, permeabilized in 0.1% (w/v) Triton X-100/PBS for 15 min, and then blocked with 5% (w/v) non-fat dried milk/PBS for 30 min. The cells were then incubated for 2 h with the anti-6B4 proteoglycan antiserum (1:500 dilution), and then with either anti-cortactin (1:100 dilution) or FITC-conjugated phalloidin (5 U/ml) for a further 2 h. The cells incubated with the anti-6B4 proteoglycan and anti-cortactin were next incubated for 60 min with Texas Red-conjugated anti-rabbit IgG (1:200 dilution) and FITC-conjugated anti-mouse IgG (1:100 dilution). For cells incubated with anti-6B4 proteoglycan and FITC-conjugated phalloidin, Texas Red-conjugated anti-rabbit IgG alone was used.

Cultured cortical neurons were fixed, permeabilized and blocked as above, and then incubated for 2 h with the anti-6B4 proteoglycan antiserum (1:500 dilution) and anti-cortactin (1:100 dilution). The cells were incubated for 30 min with FITC-conjugated anti-mouse IgG (1:100 dilution) and biotinylated anti-rabbit Ig (1:200 dilution), followed by incubation for 30 min with Texas Red Avidin D (1:1,000 dilution).

The cells were mounted and observed under a Zeiss fluorescence microscope. All solutions were diluted with 5% (w/v) non- fat dried milk/PBS, and all incubations were carried out at room temperature.

RESULTS

Identification of PTPζ Isoforms as Chondroitin Sulfate Proteoglycans—Previously, we demonstrated the presence of multiple proteoglycan-type PTPs including PTPζ-A in rat brain (P8) on the combined use of DEAE-Toyopearl column chromatography and CsCl density gradient centrifugation, which separate proteoglycans from normal proteins (*7*). In this study, we first applied the same procedure to P0 and P20 rat brains to further characterize PTPζ isoforms developmentally. Figure 1 shows the results of CsCl density gradient centrifugation. Developmental changes in the sedimentation pattern of PTP activity were clearly observed. Total PTP activity recovered in the high

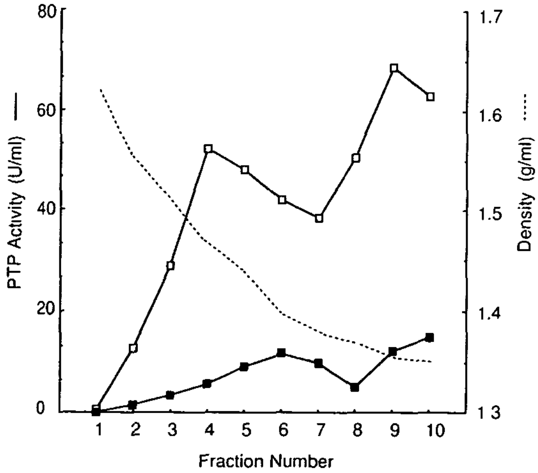

Fig. 1. **Purification of proteoglycan-type PTPs.** CHAPS-extracts of postnuclear fractions from P0 and P20 rat brains were applied to a DEAE-Toyopearl column, and the proteins were eluted with 0.6 M NaCl after washing with 0.25 M NaCl. The eluted proteins were fractionated by CsCl density gradient centrifugation. The samples were fractionated into 10 tubes, and the density (----) and PTP activity (□, P0; ■, P20) of each fraction were measured. [^{32}P]Phosphotyrosine-labeled Raytide was used as a substrate for the assaying of PTP activity.

density fraction ($\rho > 1.35$ g/ml; proteoglycan-type PTPs) decreased markedly at P20 in comparison with at P0, and the peak position of proteoglycan-type PTP activity shifted to the lower density fraction (from fraction number 4 at P0 to fraction number 6 at P20).

Aliquots of the proteoglycan fraction from P0 rat brain were analyzed first using three kinds of antibodies against PTPζ; anti-6B4 proteoglycan, antiserum 31-5, and anti-RPTPβ (Fig. 2A). Anti-6B4 proteoglycan is an antiserum raised against purified PTPζ-S (6B4 proteoglycan), the epitopes of which are shared by the three isoforms of PTPζ (7). Antiserum 31-5 recognizes the C-terminal region of PTPζ-S, which is also present on PTPζ-A but not on PTPζ-B, as this region is deleted in PTPζ-B (7). Anti-RPTPβ is a monoclonal antibody against D2 domain of PTPζ/RPTPβ, and should recognize PTPζ-A and -B. The proteoglycan fraction was subjected to immunoprecipitation with the anti-6B4 proteoglycan antibody which recognizes all the three isoforms (Fig. 2B). When the precipitate was treated with chondroitinase ABC, several core proteins, mainly 220, 300, and 380 kDa ones, were detected. Previously, we demonstrated that the 300 and 380 kDa core proteins correspond to those of PTPζ-S and PTPζ-A, respectively (7). A chondroitin sulfate proteoglycan with 220 kDa core protein was recognized by anti-RPTPβ but not by antiserum 31-5, indicating that this proteoglycan has D2 domain and at least a part of the extracellular segment of PTPζ. Furthermore, transfection of the PTPζ-B expression plasmid into mouse L cells resulted in the expression of a chondroitin sulfate proteoglycan with 220 kDa core protein (see below). These results indicated that the proteoglycan with 220 kDa core protein is PTPζ-B.

When aliquots of all the fractions obtained on CsCl density gradient centrifugation from P0 rat brain were analyzed, the anti-6B4 proteoglycan recognized the 220, 300, and 380 kDa core proteins (Fig. 3A), whereas antiserum 31-5 reacted only with the 300 and 380 kDa core proteins (Fig. 3B), and anti-RPTPβ with the 220 and 380 kDa core proteins (Fig. 3C), reproducibly. The 300 kDa core protein band was detected in the higher density fractions than the others, suggesting that PTPζ-S is the isoform most highly modified by chondroitin sulfate.

Next, aliquots of the fractions from P20 rat brain were analyzed with anti-RPTPβ after chondroitinase ABC digestion (Fig. 3D). In contrast to the results for P0 brain, the 380 kDa core protein of PTPζ-A was detected only faintly, and an intense band of the 220 kDa core protein of PTPζ-B was detected around fraction number 6. These results suggest that the expression of PTPζ is dynamically regulated during brain development with regard to carbohydrate modification and RNA splicing.

Developmental Changes in the Expression of the PTPζ Isoforms—Developmental expression of the PTPζ isoforms was further examined in the brain from E13 to P52. When brain homogenates were analyzed by Western blotting using antiserum 31-5, only the 300 kDa core protein of PTPζ-S was detected during development, indicating that PTPζ-S is far more abundant than PTPζ-A (Fig. 4A). The expression of PTPζ-S increased from E13 to the perinatal period, and then gradually decreased thereafter. Next, the developmental expression of PTPζ-A and -B was examined using anti-RPTPβ (Fig. 4B). Expression of the 380 kDa core protein of PTPζ-A was maintained from E13 to P0, and then markedly decreased and became almost undetectable after P20. Compared with the other isoforms, little change was observed in the expression level of PTPζ-B during development. However, unexpectedly in the prenatal period, non-proteoglycan-type PTPζ-B was detected. Especially at E13-15, a sharp 220 kDa band was detected without chondroitinase ABC digestion, indicating that a substantial amount of this isoform was not modified by chondroitin sulfate at this early stage (Fig. 4B). Non-proteoglycan-type PTPζ-B decreased from E13 to P0, and after P0, almost all of this isoform was expressed as a chondroitin sulfate proteoglycan. These results indicate that chondroitin sulfate modification of PTPζ is developmentally regulated in an isoform-specific manner.

Differences in the Carbohydrate Modification of PTPζ Isoforms—PTPζ-S is modified with $\mathrm{Le^x}$, HNK-1 carbohydrate, and keratan sulfate (*31-33*). PTPζ-A has also been shown to contain HNK-1 carbohydrate in rat brain (7), and keratan sulfate in chick brain (*28*). To further clarify the carbohydrate modification patterns of PTPζ isoforms, aliquots of fraction numbers 5 and 6 obtained on CsCl density gradient centrifugation from P0 rat brain were subjected to immunoprecipitation with the anti-6B4 proteoglycan, and the immunoprecipitates were analyzed by Western blotting using monoclonal antibodies to carbohydrates. As shown in Fig. 5, the 380 kDa core protein of

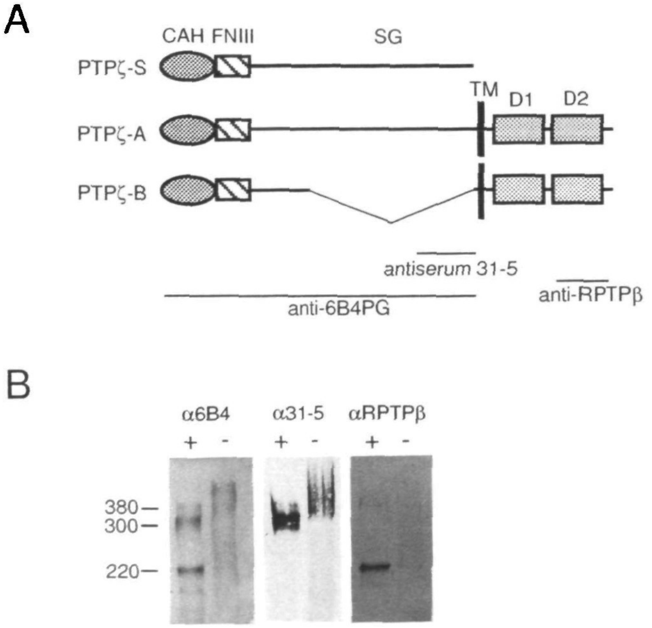

Fig. 2. **Identification of three PTPζ isoforms in the proteoglycan fractions.** (A) Schematic representation of the PTPζ isoforms and antigenic regions of the antibodies. CAH, carbonic anhydrase-like domain; FNIII, fibronectin type III domain; SG, serine-glycine-rich region (the 853 amino acid sequence deleted in rat PTPζ-B); TM, transmembrane segment; D1, D2, tyrosine phosphatase domains. (B) The immunoprecipitate with the anti-6B4 proteoglycan antibody contained three PTPζ isoforms. Aliquots of the CsCl gradient fractions (numbers 4-6) from P0 rat brain were subjected to immunoprecipitation with the anti-6B4 proteoglycan, and the immunoprecipitate was analyzed by immunoblotting using the anti-6B4 proteoglycan (α6B4), antiserum 31-5 (α31-5), and anti-RPTPβ (αRPTPβ). The positions of the core proteins of PTPζ-A (380), -S (300), and -B (220) are shown on the left in kDa. +, chondroitinase ABC-treated samples; −, nontreated samples.

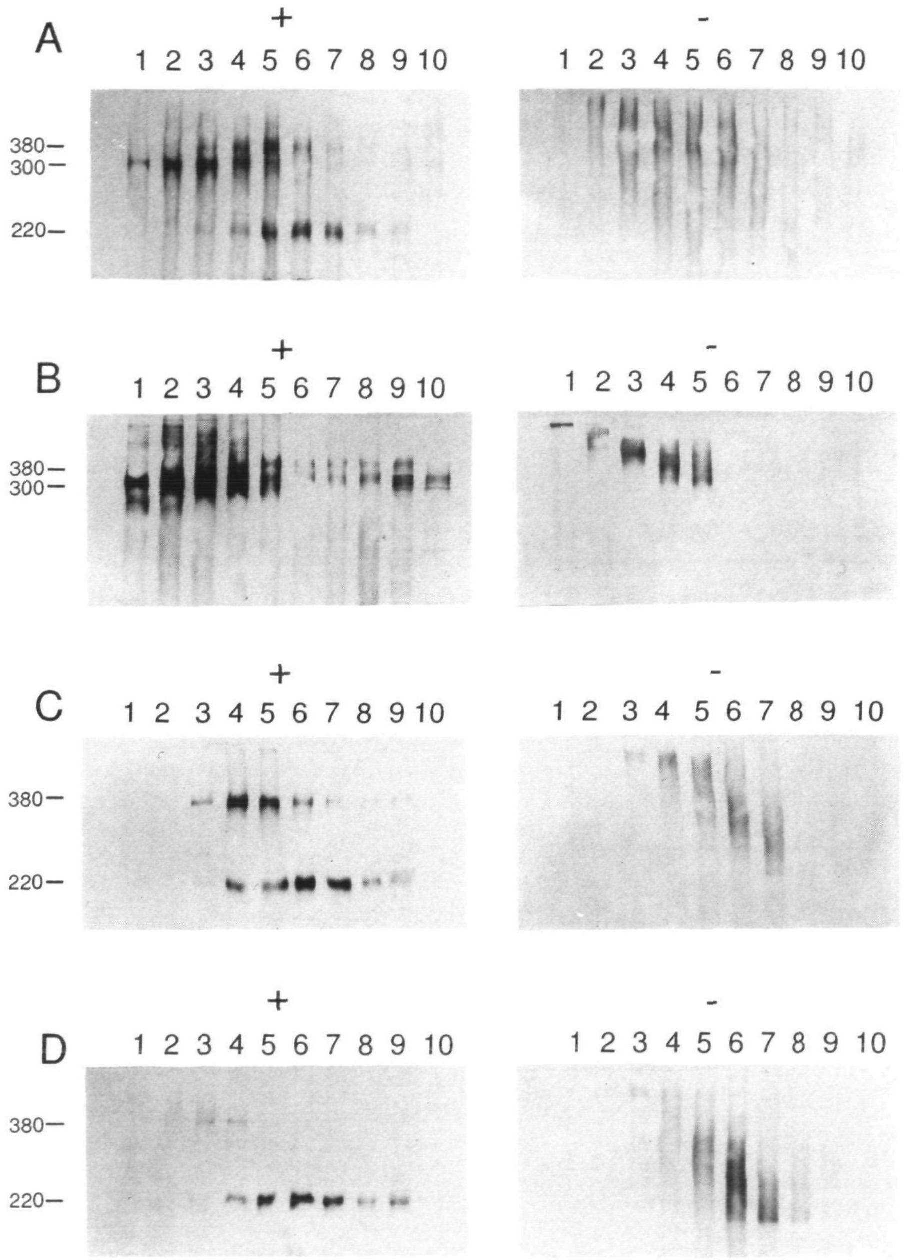

Fig. 3. **Developmental characterization of the three PTPζ isoforms.** Aliquots of the fractions obtained on CsCl density gradient centrifugation from P0 (A-C) and P20 (D) rat brains were subjected to 5% SDS-PAGE before (−) or after (+) chondroitinase ABC digestion. The samples were analyzed by immunoblotting using the anti-6B4 proteoglycan (A), antiserum 31-5 (B), and anti-RPTPβ (C, D). The positions of the core proteins of PTPζ-A (380), -S (300), and -B (220) are shown on the left in kDa.

PTPζ-A was recognized by monoclonal antibodies against Le^x, HNK-1 carbohydrate, and keratan sulfate. In contrast, the 220 kDa core protein of PTPζ-B was recognized by none of these antibodies. Essentially the same results were obtained for samples from P8 rat brains (data not shown). These results indicated that PTPζ-A is also modified with Le^x, HNK-1 carbohydrate, and keratan sulfate, similarly to PTPζ-S, but that PTPζ-B is not modified with these carbohydrates.

Characterization of cDNA-Derived PTPζ-A and -B Expressed in L Cells—Various receptor-like protein tyrosine phosphatases are localized in specific subcellular regions such as focal adhesions (*34*). Due to the lack of antibodies which recognize each of these PTPζ isoforms selectively, we could not determine the localization of the PTPζ isoforms within cells. To overcome this difficulty, we prepared L cell transfectants stably expressing cDNA-derived PTPζ-A and -B.

The expressed proteins were first analyzed by immunoblotting using the anti-6B4 proteoglycan antiserum (Fig. 6A). As in the brain samples, PTPζ-A and -B were expressed in the L cells as chondroitin sulfate proteoglycans with 380 and 220 kDa core proteins, respectively. In the PTPζ-A-producing cells, large amounts of 125-150 kDa proteins were also detected with the anti-6B4 proteoglycan (Fig. 6A, arrowhead), which were not present in the cells transfected with the PTPζ-B plasmid (Fig. 6A) or the vector only (data not shown). These proteins were not recognized by antiserum 31-5 or anti-RPTPβ (data not shown), suggesting that they are immature or processed molecules lacking the C-terminal portion of PTPζ-A. The morphology and growth rate of the transfectants expressing PTPζ-A and -B were not different from those of the mock transfected cells (data not shown).

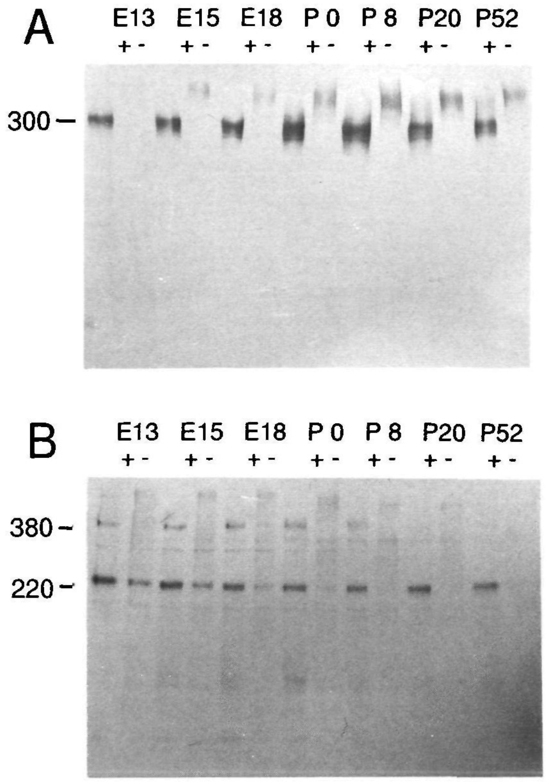

Fig. 4. **Developmental expression of the PTPζ isoforms.** (A) Brain homogenates prepared from E13 to P52 rats were solubilized with 0.2% Triton X-100 and 0.1% sodium deoxycholate. After centrifugation at 15,000 × *g* for 15 min at 4°C, the supernatants were analyzed by immunoblotting using antiserum 31-5 before (−) or after (+) chondroitinase ABC digestion. (B) Brain extracts prepared as described under "MATERIALS AND METHODS" were subjected to immunoprecipitation with the anti-6B4 proteoglycan. The immunoprecipitates were analyzed by immunoblotting using anti-RPTPβ before (−) or after (+) chondroitinase ABC digestion. The positions of the core proteins of PTPζ-A (380), -S (300), and -B (220) are shown on the left in kDa.

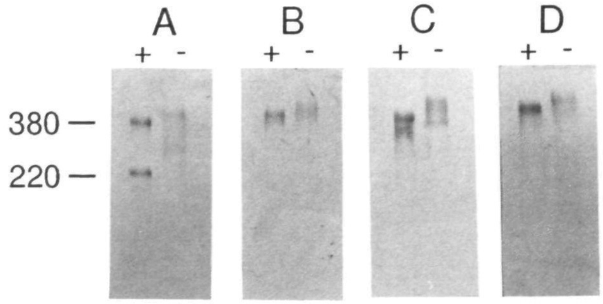

Fig. 5. **Differential carbohydrate modification of PTPζ-A and -B.** Aliquots of fraction numbers 5 and 6 obtained on CsCl density gradient centrifugation of P0 rat brain were subjected to immunoprecipitation with the anti-6B4 proteoglycan. The immunoprecipitates were analyzed by immunoblotting using anti-RPTPβ (A), HNK-1 (B), anti-Lewis X (C), and anti-keratan sulfate (D) before (−) or after (+) chondroitinase ABC digestion.

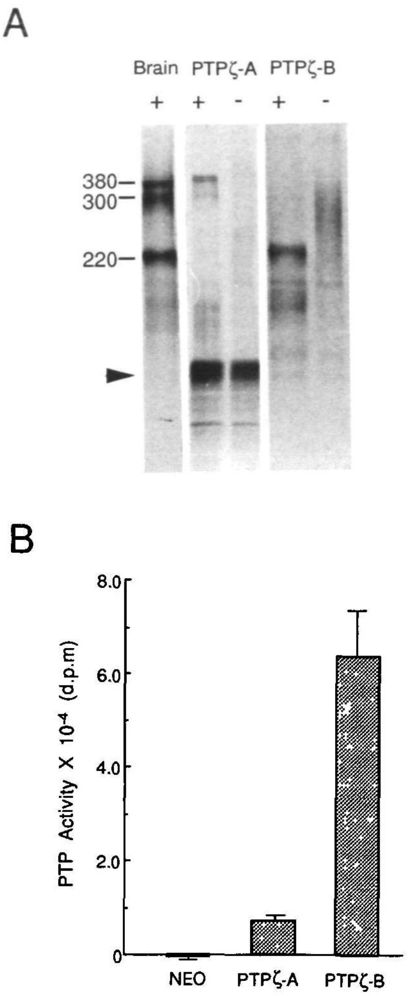

Fig. 6. **Identification and PTP activities of the cDNA-derived PTPζ-A and -B expressed in L cells.** (A) The proteins in CHAPS extracts of L cell transfectants were concentrated with DEAE-Toyopearl and then subjected to 5% SDS-PAGE before (−) or after (+) chondroitinase ABC digestion. Samples from L cells expressing PTPζ-A or -B were analyzed by immunoblotting using the anti-6B4 proteoglycan. As positive controls, aliquots of fraction numbers 5 and 6 obtained on CsCl density gradient centrifugation of P8 rat brain were analyzed by immunoblotting using the anti-6B4 proteoglycan after chondroitinase ABC digestion (brain). The positions of the core proteins of PTPζ-A (380), -S (300), and -B (220) are shown on the left in kDa. The arrowhead indicates the 125-150 kDa protein detected in the PTPζ-A-producing cells. (B) CHAPS extracts were prepared from L cells expressing PTPζ-A and PTPζ-B, and the cells transfected with the vector alone (NEO). The extracts were subjected to immunoprecipitation with the anti-6B4 proteoglycan antibody or preimmune rabbit IgG. The immunoprecipitates were incubated with $[^{32}P]$phosphotyrosine-labeled Raytide for 60 min at 30°C. The PTP activity of each transfectant was obtained as the difference in the radioactivity of released phosphate between the immunoprecipitates with the anti-6B4 proteoglycan and preimmune rabbit IgG ($n=3$).

Next, CHAPS extracts of these transfectants were subjected to immunoprecipitation using the anti-6B4 proteoglycan antiserum to measure the PTP activities of the expressed PTPζ-A and -B. Both immunoprecipitates showed a significant amount of PTP activity with $[^{32}P]$-phosphotyrosine-labeled Raytide as a substrate (Fig. 6B). The PTP activity of PTPζ-B-producing cells was reproducibly 3- to 10-fold higher than that of PTPζ-A-producing cells.

Immunohistochemical Localization of PTPζ-A and -B in the L Cell Transfectants—To determine the subcellular localization patterns of PTPζ-A and -B, we immunohisto-

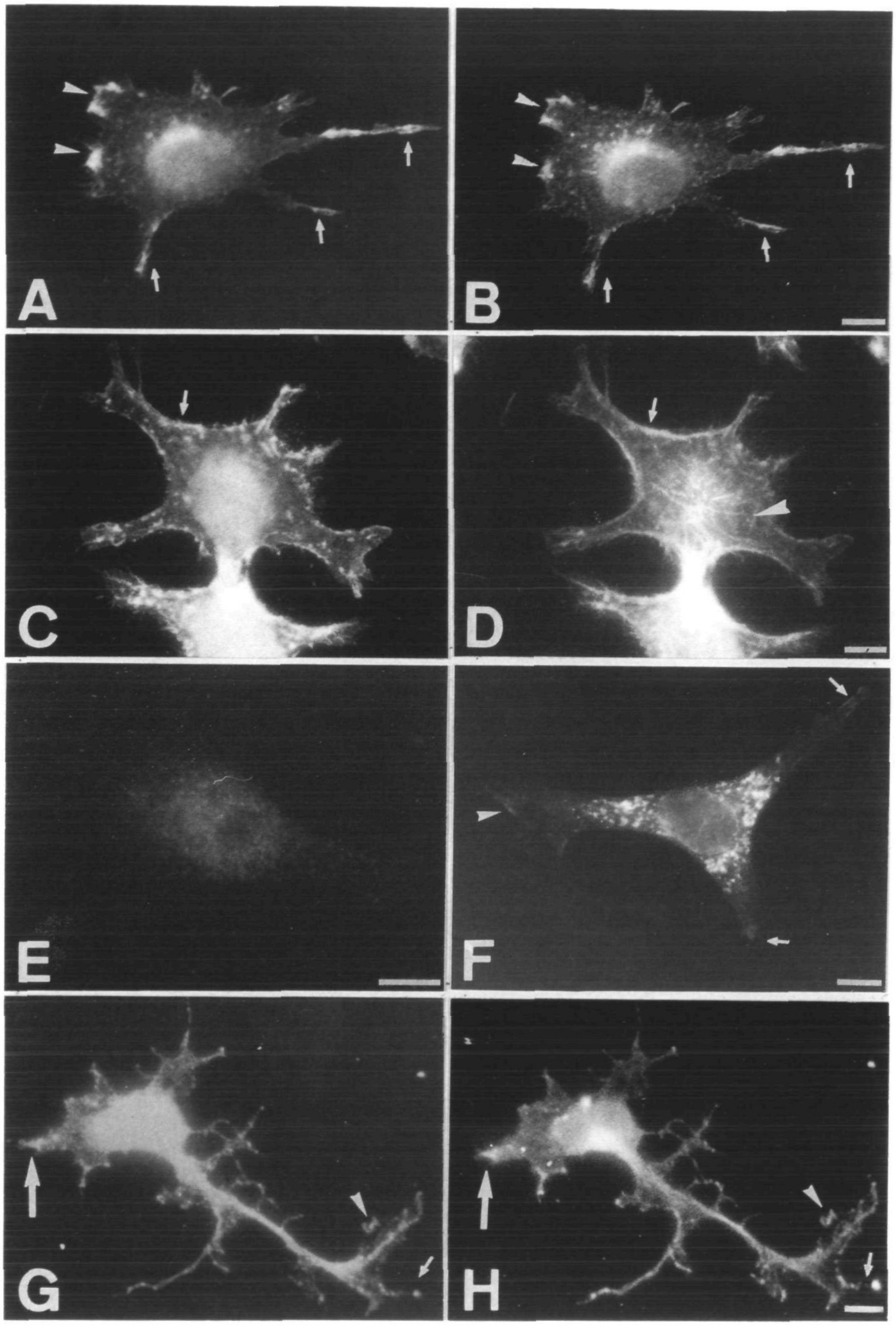

Fig. 7. **Immunohistochemical localization of PTPζ in the L cell transfectants and cortical neurons.** L cell transfectants expressing PTPζ-B were double immunostained with the anti-6B4 proteoglycan (A) and anti-cortactin (B), or the anti-6B4 proteoglycan (C) and FITC-phalloidin (D). PTPζ-B and cortactin were colocalized in the processes (arrows in A and B) and ruffling membranes (arrowheads in A and B). PTPζ-B was distributed along F-actin at the cell periphery (arrows in C and D), but was not observed on stress fibers (arrowhead in D). When rabbit preimmune serum was used instead of the primary antibodies, only weak background staining was observed (E). In the PTPζ-A-producing cells, the processes (arrows) and ruffling membranes (arrowhead) were stained with the anti-6B4 proteoglycan, although strong immunoreactivity was also observed inside the cells (F). Cortical neurons from E16 rats were cultured overnight on poly-L-lysine-coated coverslips, fixed, and then double-stained with the anti-6B4 proteoglycan (G) and anti-cortactin (H). The anti-6B4 proteoglycan epitope and cortactin were colocalized in the growth cones (arrowheads) and filopodial processes (small arrows), and at the cell periphery (large arrows). Bars, 20 μm.

chemically stained the L cell transfectants with the anti-6B4 proteoglycan (Fig. 7). Indirect immunofluorescence analysis of PTPζ-B-producing cells with the anti-6B4 proteoglycan showed strong staining in the membrane ruffles and processes (Fig. 7A). The actin-binding protein, cortactin, is abundant in cell cortical structures such as lamellipodia and ruffling membranes (*35*), and therefore we performed double immunostaining experiments. As shown in Fig. 7, A and B, PTPζ-B and cortactin were colocalized in the ruffling membranes and processes. Double immunostaining with FITC-phalloidin and the anti-6B4 proteoglycan revealed that PTPζ-B was distributed along the F-actin at the cell periphery (Fig. 7, C and D). However, PTPζ-B was not observed on stress fibers (Fig. 7, C and D). In the PTPζ-A-producing cells, the ruffling membranes and processes were again stained with the anti-6B4 proteoglycan antiserum (Fig. 7F), although the staining was not as strong as that in the PTPζ-B-producing cells. In addition to this, strong immunostaining was observed inside of the cells, probably due to the accumulation of immature or processed products of PTPζ-A.

Double immunofluorescence of cultured cortical neurons also revealed the colocalization of the anti-6B4 proteoglycan-epitope and cortactin in the growth cones and filopodial processes, and at the cell periphery (Fig. 7, G and H).

DISCUSSION

This is the first demonstration that PTPζ-B is a chondroitin sulfate proteoglycan, and that the expression and carbohydrate modification of PTPζ isoforms are differentially regulated during brain development. In a previous study involving protein renaturation experiments, we found that the rat brain contains at least two species of proteoglycan-type PTP, one of which is PTPζ-A (*7*). Another major PTP activity was detected for a proteoglycan core protein of 170 kDa, which was apparently different from PTPζ-B. On the other hand, little activity was detected at the position of the 220 kDa core protein of PTPζ-B, although the content of this isoform was comparable to that of PTPζ-A. This might be explained by the inferior renaturation rate since the recovery of PTP activity on renaturation is highly variable, depending on the PTP species (*36*).

The peak position and total activity of PTP recovered in high density fractions on CsCl density gradient centrifugation changed evidently during development of the brain (Fig. 1). Immunoblotting analysis indicated that PTPζ-A and -B expression was differentially regulated during development (Fig. 4). The expression of PTPζ-A was relatively constant from E13 to P0, and then markedly decreased after birth. In contrast, a substantial amount of PTPζ-B was detected in the adult brain, although PTPζ-A was almost undetectable in the brain after P20. On CsCl density gradient centrifugation, PTPζ-B was recovered in a lower density fraction than PTPζ-A (Fig. 3). Thus, the decrease in PTP activity and the shift of the peak position of proteoglycan-type PTP between P0 and P20 seen on CsCl density gradient centrifugation can be explained mainly by a decrease in the expression of PTPζ-A during this developmental period.

Another important difference was observed in the carbohydrate modification patterns of PTPζ-A and -B. In the early prenatal period (E13), a substantial amount of non-proteoglycan-type PTPζ-B was detected, which subsequently decreased, and at P0 most of the PTPζ-B was expressed as a chondroitin sulfate proteoglycan (see Fig. 4B, and compare the right and left panels in Fig. 3, C and D). In contrast, PTPζ-A and -S were continuously expressed as chondroitin sulfate proteoglycans (Fig. 4). Recently, Sakurai *et al.* reported that PTPζ-B was not expressed in the form of a proteoglycan in C6 glioma cells (*9*). We examined cDNA-derived PTPζ-B in Neuro2a neuroblastoma cells, and also found that this isoform was not modified with chondroitin sulfate chains in this cell line (data not shown). On the other hand, L cell transfectants producing cDNA-derived PTPζ-B expressed this isoform as a chondroitin sulfate proteoglycan (Fig. 6A). The serine-glycine-rich region of PTPζ is considered to be the chondroitin sulfate attachment region. PTPζ-B still contains ten Ser-Gly and Gly-Ser sequences in the corresponding region, and they may serve as attachment sites for chondroitin sulfate, which is developmentally regulated and highly cell-type dependent. On the other hand, PTPζ-A and -S contain additional sixteen Ser-Gly and Gly-Ser sites, some of which are likely to be attached to chondroitin sulfate chains constantly.

Recently, we reported that PTPζ-S binds pleiotrophin with high affinity, and chondroitin sulfate chains constitute part of the pleiotrophin-binding site on this molecule (*27*) because the binding affinity was affected by chondroitinase ABC digestion. The total binding of PTPζ-S to pleiotrophin was also decreased by ~40% after chondroitinase digestion. Milev *et al.* (*26*) demonstrated that TAG-1/axonin-1 binds to phosphacan (PTPζ-S) and neurocan. The binding of TAG-1 to phosphacan decreased by ~70% on chondroitinase ABC digestion, whereas its binding to neurocan was not affected by the same procedure. These results indicate that chondroitin sulfate chains play significant roles for some ligand molecules in the regulation of binding to PTPζ. In this context, developmentally regulated modification of PTPζ-B with chondroitin sulfate is physiologically important, and this regulation might play a more critical role in the early prenatal period, because PTPζ-B is the major transmembrane form and is present mostly as a non-proteoglycan-type molecule during this period.

We recently cloned cDNAs encoding RPTPγ isoforms from rat brain, a receptor-like PTP closely related to PTPζ (*37*). Like PTPζ, an extracellular variant of RPTPγ (RPTPγ-S) derived on RNA splicing was also identified, and it was confirmed that this molecule was secreted into the culture medium when expressed in COS7 cells. However, RPTPγ isoforms were not expressed as proteoglycans in COS7 or C6 glioma cells in contrast to PTPζ-A and -S (*37*), both of which were expressed as proteoglycans in these cells. These results suggest that ligand binding to PTPζ and RPTPγ is differentially regulated by carbohydrates despite the close similarity in the extracellular core protein structures.

PTPζ-A and -S are modified with Le^x, HNK-1 carbohydrate, and keratan sulfate, but PTPζ-B bears none of these carbohydrates (Fig. 5). The 853 amino acid region deleted in PTPζ-B contains six potential asparagine-linked carbohydrate attachment sites, which may be modified with these carbohydrates in PTPζ-A and -S. Le^x and HNK-1 carbohydrate have been suggested to be involved in cell-cell recognition (*33, 38, 39*), and keratan sulfate has a repulsive effect on some types of neurons (*40, 41*). Recently, we demonstrated that keratan sulfate modification of PTPζ-A and -S is quite strictly regulated in several boundary regions of the brain (*28*). At the mes-metencephalic boundary for instance, keratan sulfate expression spatiotemporally corresponded well to formation of the fovea isthmi, a groove separating the mesencephalon and metencephalon. Thus, differential carbohydrate modification of PTPζ isoforms might comprise another mechanism of regulation of PTPζ functions.

We produced L cell transfectants expressing cDNA-derived PTPζ-A and -B. These PTPζ isoforms were expressed in L cells as enzymatically active chondroitin sulfate proteoglycans (Fig. 6). The functions of the diverse set of PTPs are inferred by their specific subcellular localization. For example, LAR was shown to be localized at sites of focal adhesion and suggested to be involved in the orchestration of cell-matrix interactions (*34*). The cell surface expression of RPTPμ was restricted to cell-cell contacts directly associated with cadherin/catenin complexes (*42*), although this association is still controversial (*43*). RPTPκ was also reported to be localized at adherence junctions forming a complex with β/γ-catenins (*44*). In this study, we demonstrated that PTPζ-B was localized at the cell periphery, especially on the ruffling membranes, of

L cell transfectants (Fig. 7). No accumulation was observed at cell-cell contact sites or focal adhesion sites. Similar results were obtained for transfectants of rat fibroblast 3Y1 cells (data not shown). Double immunostaining with FITC-phalloidin and the anti-6B4 proteoglycan indicated that PTPζ-B was distributed along the cortical actin fibers but not along the stress fibers. Double immunofluorescence analysis also indicated that PTPζ-B was colocalized with cortactin, an actin-binding protein specifically expressed on cell cortical structures such as ruffling membranes. Cortactin is highly tyrosine phosphorylated in v-*src*-transformed cells, and is considered to transduce signals from the cell surface to the cytoskeleton (*35, 45*). Cortactin has an SH3 domain at its C-terminus. SH3 domains recognize proteins containing proline-rich sequences with at least one PXXP motif (*46*). PTPζ has a proline-rich sequence in its intracellular domain (PPTPIFPI, amino acid residues 1686-1693), which might be a binding site for cortactin. Interestingly, cultured cortical neurons also showed colocalization of cortactin and PTPζ-antigenicity, especially at the growth cones. Since the anti-6B4 proteoglycan does not distinguish the PTPζ isoforms, we were unable to determine which isoform contributed to the staining in the growth cones. However, this raises the possibility that PTPζ acts as a receptor for extracellular molecules which regulate the organization of the cytoskeleton in the growth cones.

The L cell transfectants producing PTPζ-A and -B showed essentially the same subcellular localization of these proteins in cell cortical structures. However, strong anti-6B4 proteoglycan immunoreactivity was also detected in intracellular patchy structures in the PTPζ-A transfectants. Immunoblotting analysis indicated that low molecular weight materials, probably processed or immature products of PTPζ-A, were accumulated in the L cells. The intracellular patchy structures did not correspond to the immunostaining of cathepsin D (data not shown), a marker protein of lysosomes (*47*). Thus, it seems likely that PTPζ-A is not present only as the intact mature form. In this context, it is also noteworthy that the level of the PTPζ-A protein is lower than that expected from the mRNA level, in comparison with PTPζ-B (Fig. 4 and see Ref. *16*). The 853 amino acids deleted in PTPζ-B might play important roles in the turn-over rate of this enzyme.

This study revealed large differences between PTPζ-A and -B in their developmental expression patterns and carbohydrate modification. Further studies are necessary to determine differences in the ligand and substrate specificities of these isoforms.

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