Cloning and Expression of Two Structurally Distinct Receptor-linked Protein-Tyrosine Phosphatases Generated by RNA Processing from a Single Gene*

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We describe here the first example of RNA processing generating two functional receptor-linked protein-tyrosine phosphatases (PTP) (protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) that are structurally distinct within their catalytic domains. Two cDNAs, PTP-P1 and PTP-PS, were isolated from rat pheochromocytoma cells, which encode two receptor-linked protein-tyrosine-phosphatases and are produced by alternative splicing and differential use of polyadenylation sites. Both cDNAs share an identical extracellular domain and a single transmembrane domain, but differ within their cytoplasmic regions: PTP-P1 contains two tandem repeated PTPase catalytic domains, whereas PTP-PS contains only the amino-terminal domain. Bacterial expression of PTPase domains of both cDNAs demonstrates that PTP-P1 and PTP-PS contain tyrosine-phosphatase activity. PTP-P1 is encoded by three transcripts of approximately 8, 6, and 4 kilobases, whereas PTP-PS is encoded by a single 4.8-kilobase transcript. PTP-P1 (6 kilobases) and PTP-PS are mainly expressed within the brain and in neuronal and endocrine cells. These data suggest that PTP-P1 and PTP-PS may be involved in neuronal function.

Protein tyrosine phosphorylation is an important mechanism to control cellular proliferation and differentiation. This mechanism requires both protein-tyrosine kinases (ATP: protein-tyrosine O-phosphotransferase, EC 2.7.1.112) and protein-tyrosine-phosphatases (PTPases); protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) to maintain a balance between tyrosine phosphorylation and dephosphorylation. The protein tyrosine kinase family, which has been extensively investigated and well characterized, can be divided into two classes, intracellular and transmembrane. Most of the PTPases can be further divided into four different types depending on different organization of extracellular domain. According to Fischer et al. (14), the PTPase family can be divided into three classes. Class I contains cytoplasmic PTPases, which are small molecules, that usually contain only a single PTPase catalytic domain. Class II contains receptor-linked PTPases with a single PTPase catalytic domain, and class III contains receptor-linked PTPases with two tandem repeated PTPase domains.

The recent discovery that the tyrosine kinase trk is the receptor for nerve growth factor highlights the importance of protein-tyrosine phosphorylation in neuronal differentiation (16–18). NGF, a soluble factor required for survival of sensory and sympathetic neurons, can transform PC12 cells from an endocrine cell phenotype to a sympathetic neuronal phenotype (19). This differentiation of PC12 cells induced by NGF has been extensively investigated, but the mechanism remains poorly understood. NGF stimulates tyrosine kinase activity by binding to the extracellular domain of its receptor and consequently activating a cascade of phosphorylation events (20). This process appears to involve p38ras, Raf-1 kinase, and MAP kinases (21, 22).

Although the importance of protein tyrosine kinases, like trk, in neuronal differentiation is well characterized, the involvement of PTPases in neuronal function and differentiation is much less clear. In Drosophila, three receptor-linked PTPases, DLAR, DPTP10D, and DPTP99A are selectively expressed in the central nervous system (23, 24). DLAR and DPTP99A are expressed on most axons, while DPTP10D is primarily localized to the anterior commissure and its junc-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L19180 and L19181.

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* The abbreviations used are: PTPase, protein-tyrosine-phosphatase; NGF, nerve growth factor; MAP, mitogen associated protein; PCR, polymerase chain reaction; p-Npp, p-nitrophenyl phosphate; kb, kilobase; bp, base pair.
tions with the longitudinal tracts. These findings suggest that receptor-linked protein-tyrosine-phosphatases may be involved in axonal outgrowth and guidance during embryonic development.

To begin to address the importance of PTPases in neuronal differentiation, we have characterized two cDNAs from PC12 cells that encode receptor-linked PTPases, PTP-P1 and PTP-PS, which are the results of alternative splicing and differential use of polyadenylation sites. PTP-P1 contains two PTPase catalytic domains, whereas PTP-PS contains only the first catalytic domain. PTP-P1 is encoded by three transcripts (8, 6, and 4 kb) and PTP-PS is encoded by a single 4.8-kb transcript. PTP-P1 (6 kb) and PTP-PS are mainly expressed in neuronal tissues and cells and in some endocrine cells. These data suggest that PTP-P1 and PTP-PS may be involved in neuronal function.

**EXPERIMENTAL PROCEDURES**

**Synthesis of a PC12 cDNA Library.—** A size-selected cDNA library (inserts larger than 2 kb) was prepared from PC12 cell mRNA by using the cDNA cloning kit from Life Technologies, Inc. (SuperScript II). A plasmid was prepared by a modified eukaryotic expression vector pSRoRSN. This plasmid is derived from pcDL-SRa (25), in which two original Sall sites were destroyed, and two new restriction sites (Sall and NotI) were created between the EcoRI site to allow directional cloning of modified cDNA inserts. The library was introduced into DH10B cells (Life Technologies, Inc.) by electroporation. The complexity of the library is 2.5 × 10^8 individual clones.

**PCR Amplification.—** A 200-ng sample of the plasmid PC12 cDNA library described above was used as a template in the polymerase chain reaction. Primed with two degenerate oligonucleotides for 35 temperature cycles. Each cycle consisted of 60 s at 94°C, 30 s at 45°C, and 60 s at 72°C in a PCR thermocycler (Perkin-Elmer Cetus). The PCR buffer was 50 mM KCl, 1.5 mM MgCl2, 0.1 mM dNTPs, 15 mM Tris-HCl, pH 8.4. The sequences of the two oligonucleotides are 5'-AAG(A)TG(T)-GTG(T)-GAC(T)-GTC-CC-3' and 5'-CC-G(C)AC(T)-CC-(A)-GTC-CG-3', respectively, which correspond to amino acids KCH-I and HCSGI(V)GR within the PTPase catalytic domain.

Screening of the PC12 cDNA Library with PC3 Probe and Isolation of Full-length Clones.—One of the PCR products (PC3) was used to screen the plasmid PC12 cDNA library (described above). The PC12 library was plated onto LB/ampicillin plates and transferred to nitrocellulose membranes in duplicate. The nitrocellulose membranes were washed with 2 x SSC, 1% SDS at 65°C for 2 h prior to hybridization. Hybridization was carried out at 43°C under 0.2 x SSC, 0.1% SDS at 65°C. To obtain full-length clones, an Accl fragment (see Fig. 1) was used as a probe to screen a rat cortex cDNA library. The screening yielded 370 bp of additional 5'-coding sequences. The remaining 5'-end sequences were then isolated by using 5'-rapid amplification of cDNA ends protocol (Life Technologies, Inc.). Briefly, a oligonucleotide corresponding to the 5'-end of the sequences was used as primer to synthesize cDNA from PC12 RNA using reverse transcriptase. The cDNA was tailed with sequences complementary to the anchor primer, PCR was performed using an internal primer upstream to the reverse transcriptase primer and the anchor primer. PCR products were then digested with Sall and Smal and subcloned into pBluescript SK+/-.

**DNA Sequencing.—** All PCR products and fragments of PTP-P1 and PTP-PS were subcloned into M13mp18 and M13mp19 (New England Biolabs) and pBluescript SK+/- (Stratagene). Both strands of each fragment were sequenced by the dideoxynucleotide chain termination method with modified T7 DNA polymerases (U. S. Biochemical Corp.) using restriction fragments and primer walks.

**Northern Blot Analysis.—** Total RNA was extracted from various tissues and cell lines as described in Fig. 6 legend by using guazidine/cesium chloride to isolate mRNAs (26). The RNA was electrophoresed in a formaldehyde, formamide, 0.8% agarose gel, blotted, and hybridized to 32P-labeled probes. The hybridization conditions for random-primer DNA probes were the same as those used in the screening of the library. For RNA probes, the hybridization temperature was 63°C.

**Bacterial Expression of PTPases.—** The PTPase domains of PTP-P1 (amino acids 875-1494) and PTP-PS (amino acids 875-1260) were amplified by the PCR using oligonucleotides specific to PTP-P1 and PTP-PS that incorporated NdeI and BamHI restriction sites to facilitate cloning into PET-15b (Novagen). The plasmids (pET-P1 and pET-PS) were over-expressed in Escherichia coli strain BL21/LyS3F (provided by Dr. R. H. Goodman, Vomull Institute, Oregon Health Sciences University). The overnight cultures of bacteria containing pET-P1 and pET-PS were diluted 1:100 in 100 ml of LB broth containing 0.4% glucose, cultured under selection with 50 μg/ml carbenicillin, and induced with 1 μM isopropylthio-β-D-galacto-

**RESULTS**

Cloning of PTP-P1 and PTP-PS.—To obtain clones for new members of the protein-tyrosine-phosphatase family, two degenerate oligonucleotides were synthesized, which correspond to the conserved amino acids KCH(P)QYW and HCSGI(V)GR within the PTPase catalytic domain. These degenerate oligonucleotides were used in a PCR, using the PC12 cDNA library described above as a template. The major PCR product was approximately 320 bp in length, which was subcloned into M13 and sequenced. After screening 12 clones, two were found to encode potentially novel members of the PTPase family. One of them, PC3, was used as a probe to screen the PC12 cDNA library. The initial screening yielded about 300 positive clones, 20 of them were analyzed by Southern blot, and further screening of these clones produced two partial clones, designated PTP-P1 and PTP-PS. To obtain 5'-end sequences encoding the full length of both PTP-P1 and PTP-PS, we used a Accl fragment of PTP-PS which is located in the 5'-end of initial partial clone (Fig. 1) as a probe to screen a rat cortex λ gt11 cDNA library (Clontech), the screening yielded eight overlap positive clones, one of them, with a largest insert, 1.6 kb, contains additional 370 bp of 5'-end coding sequences for both PTP-P1 and PTP-PS.

**Fig. 1. Restriction map of pPS- and PTP-P1.** Ac, Accl; H, HincII; S, SacI; Sm, Smal; A, Apal; K, Kpnl. The gray boxes represent the transmembrane domains and the black boxes represent the PTPase catalytic domains. The lines correspond to the restriction map and the boxes represent the fragments used as probes for Northern blot hybridization.
Amino acids of PTP-PS are shown. The putative polyadenylation signal is 4303-5979 and its predicted amino acid sequences 1235-1494.

Nucleotide sequences 1-4302 and its predicted amino acid sequences shared by PTP-P1 and PTP-PS are shown. The potential signal peptide and transmembrane domain are

[underlined text]

Fig. 2. Nucleotide and amino acid sequences of PTP-PS and PTP-P1. A, sequences shared by both PTP-1 and PTP-PS. Nucleotide sequences 1-4302 and its predicted amino acid sequences shared by both PTP-1 and PTP-PS are shown. The potential signal peptide and transmembrane domain are underlined. B, sequences unique to PTP-PS. Nucleotide sequences 4303-4419 and its predicted unique amino acid sequences 410-922 are shown.
PS. The remaining 5'-end sequences of both PTP-P1 and PTP-PS were then isolated using a 5'-rapid amplification of cDNA ends protocol.

Both PTP-P1 and PTP-PS P1 share amino acid residues 1-1234 and nucleotide sequences 1-4302 (Fig. 2A) encoding a potential signal peptide (the first 23 residues), an extracellular domain of 823 amino acids, and a single transmembrane domain. The extracellular domain contains two IgG-like domains and four fibronectin type three repeats (32).

In addition, PTP-P1 contains two PTPase catalytic domains within its cytoplasmic region and a 900-bp A-T rich 3'-untranslated region (Fig. 2C). In contrast, PTP-PS contains a single NH2-terminal PTPase catalytic domain and a unique carboxy-terminal sequence of 26 amino acids (residues 1235-1260, Fig. 2B) and a very short 3'-untranslated region (Fig. 2B).

PTP-P1 and PTP-PS are highly homologous to LAR (33) and human PTPase-6 (15) in their catalytic domains (Fig. 3). LAR and human PTPase-6 encode a potential signal peptide (the first 23 residues), an IgG-like domain and four fibronectin type three repeats (32).

Northern blots were performed to analyze the expression of PTP-P1 and PTP-PS. A DNA probe (Probe I) prepared from a fragment of PTP-P1 (generated by SacI and KpnI digestion (1.07 kb) and covering the first catalytic domain) (Fig. 1) that is shared by both clones, detected four discrete transcripts, which were approximately 8, 6, 4, and 4 kb (Fig. 4A). Another DNA probe (Probe 2, Fig. 1) prepared from a fragment (containing the carboxyl-terminal portion of the second catalytic domain and the 3'-untranslated region of PTP-P1) specific to PTP-P1, detected three discrete transcripts, which were 8, 6, and 4 kb long (Fig. 4A).

An RNA probe (Probe 3, Fig. 1) derived from sequences unique to the 3'-end of PTP-PS, detected a single 4.8-kb transcript (Fig. 4A).

**Fig. 2—continued**
FIG. 3. Homology between PTP-P1, HPTP-6, and LAR. The amino acid sequences of two tandem repeated PTPase domains are boxed. The single letters are used. The transmembrane domains are highlighted by boldface letters.

FIG. 4. Messenger RNA expression in PC12 cells, detected by Northern blot hybridization. A, hybridization with a probe (Probe 1) shared by both PTP-P1 and PTP-PS. B, hybridization with a probe (Probe 2) specific to PTP-P1. C, hybridization with an RNA probe (Probe 3) specific to PTP-PS. The PTP-P1 probes were labeled with '*P by random priming. The sequences unique to the PTP-PS 3'-end were subcloned into pBluescript SK+ and transcribed in vitro by T7 RNA polymerase. The PCR products, shown in Fig. 5B, were subcloned into M13 and sequenced. The results demonstrate that PTP-P1 and PTP-PS are alternative spliced transcripts of a single gene. Excision of the intron containing the sequence unique to PTP-P1 and the 63-bp intronic sequence produces PTP-P1, whereas PTP-PS is produced by differential use of the polyadenylation site that is included within this intron. The consensus intron donor and acceptor sites, polyadenylation site, and splicing pattern are shown in Fig. 5C. The unique 26 amino acids at the 3'-end of PTP-PS shares no significant homology with known proteins.

Gene Expression of PTP-P1 and PTP-PS in Tissues and Cells—A number of tissues and cell lines were examined for gene expression of PTP-P1 and PTP-PS. The PTP-P1 probes were labeled with 32P by random priming. The sequences unique to the PTP-PS 3'-end were subcloned into M13 and transcribed in vitro by T7 RNA polymerase in the presence of [α-32P]CTP. The PCR products, shown in Fig. 5B, were subcloned into M13 and sequenced. The results demonstrate that PTP-P1 and PTP-PS are alternative spliced transcripts of a single gene. Excision of the intron containing the sequence unique to PTP-P1 and the 63-bp intronic sequence produces PTP-P1, whereas PTP-PS is produced by differential use of the polyadenylation site that is included within this intron. The consensus intron donor and acceptor sites, polyadenylation site, and splicing pattern are shown in Fig. 5C. The unique 26 amino acids at the 3'-end of PTP-PS shares no significant homology with known proteins.
FIG. 5. PTP-P1 and PTP-PS are generated by RNA processing. A, schematic map of the PCR strategy used. D1 stands for PTPase domain 1, D2 stands for PTPase domain 2, the light gray region stands for PTP-PS 3′-end unique sequence. B, the PCR amplification of genomic DNA. The photograph represents ethidium bromide-stained gel demonstrating the position of amplified PC12 genomic DNA using the primers shown. Lane 1, DNA marker; lane 2, product of primers 1 and 2; lane 3, product of primers 1 and 3. C, intronic and junctional sequences located between the two PTPase domains of PTP-P1. The splice donor site and the splice acceptor site are in dark gray boxes, the sequences unique to the 3′-end of PTP-PS is enclosed within a light gray box. Intronic sequences are shown in lower case letters.

Fig. 6. Messenger RNA expression of PTP-P1 and PTP-PS in tissues and cells, detected by Northern blot hybridization. A, hybridization with a DNA probe shared by both PTP-P1 and PTP-PS (Probe 1). Hybridization of the same blot to human β-actin DNA probes are shown in the bottom. B, hybridization with an RNA probe specific to PTP-PS (Probe 3). The 28 S ribosomal band of the same filter stained with methylene blue is shown in the bottom. Cell line and tissues are noted in the figure. Abbreviations used include: W2, rat medullary thyroid carcinoma; COS, monkey kidney cells; Molt-B1, human B lymphocytes; Hela, human epithelial carcinoma; MIA PaCa-2, human pancreatic carcinoma; SKN-MC, human neuroblastoma; HT-29, human colonic carcinoma; LX-1, human small lung carcinoma; Rin 5F, rat insulinoma; GH4C3, rat pituitary tumor; PC12, rat pheochromocytoma.
We have characterized two cDNAs from PC12 cells, PTP-P1 and PTP-PS, that encode receptor-linked protein-tyrosine-phosphatases. The two phosphatases are generated by alternative splicing and differential use of polyadenylation sites. PTP-P1 and PTP-PS are highly homologous to HTP-6 (15) and LAR (33) in their catalytic domains, indicating that they are members of the "LAR-like" subfamily of receptor-linked protein-tyrosine-phosphatases. Bacterial expression studies demonstrate that PTP-P1 and PTP-PS contain protein-tyrosine-phosphatase activity (33, 34).

Using a probe specific to PTP-P1 (Probe 2, Fig. 1), we detected three transcripts which are approximately about 8, 6, and 4 kb in length. These three transcripts were also detected when using a probe common to both PTP-P1 and PTP-PS (Probe 1, Fig. 1). These results suggest that the three PTP-P1 transcripts all share two catalytic domains and/or 3'-untranslated region but differ in 5'-end sequences that encode the extracellular domains of these proteins. Similar heterogeneity within the extracellular domains of neural cell adhesion molecule and the phosphatase CD45 are generated by alternative splicing (35, 36). Therefore, we suggest that the heterogeneity of transcripts encoding PTP-P1 may also arise by alternative splicing within the extracellular domain. In contrast, PTP-PS is encoded by a single transcript that is generated by RNA processing within the cytoplasmic domain.

This family of PTPases represents the first example of RNA processing generating PTPases of two structurally distinct classes. The excision of an intron that separates the two catalytic domains produces PTP-P1. The inclusion of this intron allows the synthesis of PTP-PS. This intron contains the COOH-terminal 26 amino acids of PTP-PS and a typical polyadenylation site. Its use allows the synthesis of PTP-PS with a single catalytic domain.

The physiological significance of this type of RNA processing remains to be characterized. Since both processed RNAs encode proteins with PTPase activity, it is possible that these two PTPases may be differentiated either by their substrate specificity or enzyme kinetics. An example of alternative splicing within the phosphatase domain was described for LRP by Matthews et al. (37). LRP is a receptor-linked protein tyrosine phosphatase encoded by a gene containing a 108-bp intron whose inclusion disrupts the first PTPase catalytic domain.

We have examined the gene expression of PTP-P1 and PTP-PS. PTP-P1 (8 kb) transcript is widely expressed, but PTP-P1 (6 kb) is mainly expressed within the brain and in neuronal cells. PTP-PS is also expressed in neuronal and endocrine cells, suggesting that both PTP-P1 (6 kb) and PTP-PS may be involved in neuronal functions. PTP-P1 and PTP-PS contain an extracellular domain with four fibronectin type III repeats and three IgG-like domains. Many receptor-linked protein tyrosine phosphatases contain several N-CAM-like fibronectin type III repeats and IgG immunoglobulin-like domains within their extracellular domains (14), these sequence elements may be involved in cell-cell interaction and cell migration during neuronal development. In addition, PTP-P1 mRNA levels are increased following NGF-induced PC12 cell differentiation. Moreover, vanadate, a specific inhibitor of PTPases, was recently shown to be able to block NGF-induced PC12 cell differentiation (38). Therefore, we speculate that PTP-P1 might be involved in neuronal differentiation. Since PTP-P1 differs from PTP-PS by its inclusion of a second PTPase domain, we suggest that this second domain may have regulatory function in vivo.

Acknowledgments—We are grateful to Dr. J. H. Wang for providing cdc2(6-20) peptide, Dr. A. Shaw for providing the plasmids pCD-SRα, pBluescript-lck, and vaccinia virus, and Dr. K. Walton for helpful discussion and sharing the sequence information prior to publication.

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RNA Processing of Novel Protein-Tyrosine Phosphatases