RESEARCH COMMUNICATION

Cloning and expression of protein tyrosine phosphatase-like protein derived from a rat pheochromocytoma cell line

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A novel protein [designated protein tyrosine phosphatase-like protein (PTPLP)] which is distantly related to receptor-type protein tyrosine phosphatases (PTPases) was cloned from a rat pheochromocytoma cell line. The PTPLP was detected exclusively in the brain. Overexpression of the PTPLP decreased the basal PTPase activity of COS-7 cells for Raytide. These results suggest that PTPLP may function as a negative regulator of PTPases in neuronal tissues.

INTRODUCTION

Cell growth and differentiation are elaborately regulated by phosphorylation and dephosphorylation of cellular proteins [1]. Tyrosine kinase activation, which is triggered by the interaction of various growth factors with their receptors, leads to sequential phosphorylation of functional proteins and causes mitogenesis and differentiation of a variety of cell types [2]. Protein tyrosine phosphatases (PTPases) counteract tyrosine kinases through their catalytic activity of dephosphorylation. As reported recently, some PTPases are involved in the transformation of rat embryo fibroblasts [3], neuronal differentiation [4] and activation of $p34^{cdc2}$ [5]. Thus PTPases as well as tyrosine kinases are thought to play critical roles in these cellular events.

PTPases are classified into cytosolic and membrane-associated forms, and the latter can be subdivided into four categories according to their structural characteristics [6]. It is interesting to note that, except for human protein tyrosine phosphatase β (HPTPβ), most PTPases are made up of two tandem PTPase catalytic domains, each of which comprises ~ 230 amino acid residues. The structures of the catalytic domains are considerably diverse except for 11 amino acid residues, [I/V]HCXAGXXR[S/T]G, located in the C-terminal catalytic region. The existence of the tandem catalytic domains does not necessarily seem to be required for the manifestation of PTPase activity [7]. As far as leukocyte common antigen (LCA) and its related protein (LAR) are concerned, the first PTPase domain is more important for their intrinsic catalytic activity, and the second one is involved in determination of substrate specificity [8]. However, a detailed molecular mechanism underlying the involvement of the two domains in the manifestation of the function is still ambiguous.

The objective of the present study was to isolate a membrane-associated PTPase expressed in a rat pheochromocytoma cell line which is known to be potentially differentiated into a sympathetic-neuron-like phenotype by nerve growth factor [9]. Using the PCR method, we could identify a protein that is structurally related to PTPases. This protein appears to be non-functional in terms of PTPase activity, but to modulate the activities of other PTPases. Thus we designated the protein as rat protein tyrosine phosphatase-like protein (PTPLP). Here we report the molecular cloning of rat PTPLP and discuss its biological significance.

EXPERIMENTAL

Materials

A pheochromocytoma cell line, PC12w, was kindly provided by Dr. Robert Speth (Washington State University) and maintained as described previously [10]. Raytide, a substrate for PTPases, and $pp60^{c\text{-}src}$ were purchased from Oncogene Science (San Diego, CA, U.S.A.). A cDNA synthesis kit and a poly(A)$^+$ RNA extraction kit were from GIBCO BRL and Invitrogen respectively. A cDNA library construction kit based on λgt10 was obtained from Amersham. [γ-^{32}P]ATP and [α-^{32}P]dCTP (111 TBq/mmol) were purchased from New England Nuclear. Other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Construction of a PC12w cDNA library

PC12w cells [1.2×10^8 cells from 15 dishes (10 cm diam.)] were washed once with PBS (4 ml/10^7 cells) and were used for poly(A)$^+$ RNA isolation. Of the RNA, 5 μg was oligo(dT)-primed and converted into the corresponding double-stranded cDNA. The cDNA was ligated with the *Eco*RI adapters provided in a λgt10 ligation kit (Amersham) and then integrated into λgt10 vector. The recombinant phage DNA was packaged and was divided into pools of ~ 10000 plaques on LB plates of 15 cm diameter.

Probe for rat PTPLP

Oligo(dT)-primed cDNA (~ 50 ng) was amplified by the PCR using degenerate sense and antisense primers, which were designed for the most conserved amino acid sequences of PTPases and contain either *Bam*HI or *Eco*RI sites at the 5′ terminus. Their sequences are 5′-CGGAATTCGAYTAYATNAAYGCY-3′

Abbreviations used: BCS, biodegradable counting scintillant; LAR, leukocyte common antigen-related protein; LCA, leukocyte common antigen; MBP, myelin basic protein; PTPase, protein tyrosine phosphatase; PTPLP, protein tyrosine phosphatase-like protein; HPRPβ, human protein tyrosine phospatase β.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank and EMBL Data Bank with the accession number D38222.

and 5′-CGCGGATCCNGGCCARTANKSNGYRCA-3′. The PCR was carried out using 2.5 units of *Taq* DNA polymerase (Promega) in 50 μl of 50 mM KCl/2.5 mM $MgCl_2$/0.1 % (v/v) Triton X-100/250 μM dNTPs/10 mM Tris/HCl (pH 9.0) containing 250 pmol of each primer (1 unit is defined as the amount of enzyme that will incorporate 10 nmol of dNTPs into acid-insoluble material in 30 min at 74 °C). Twenty-five cycles of the PCR were performed; each was carried out at 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1.5 min. After digested with *Bam*HI and *Eco*RI, the PCR products were subcloned into pBluescript KS(+) using DH5α and sequenced. After liquid culture (100 ml), the plasmid DNA was isolated by alkali lysis followed by precipitation with polyethylene glycol 6000 [11]. The cDNA insert was excised with the restriction enzymes and was purified by 1.5% (w/v)-agarose-gel electrophoresis. Approx. 50 ng of the DNA fragment was labelled with [α-^{32}P]dCTP using a Prime-It kit (Strategene) and was used for probing every time.

Combination 1

	Sequences	Primers
Sense	-DYINA-	5′ -CGGAATTCGAYTAYATNAAYGCY-3′
Antisense	-CxxYWP-	5′ -CGCGGATCCNGGCCARTANKSNGYRCA-3′

Combination 2

	Sequences	Primers
Sense	-CxxYWP-	5′ -CGGAATTCAARTGYSNNSARTAGGCC-3′
Antisense	-VHCSAGV-	5′ -CGCGGATCCCCNCYNGCNGARCARTGNAC-3′

Figure 1 Primers used in the polymerase chain reaction

Highly conserved amino acids and their corresponding oligonucleotide sequences of the primers employed for the PCR are shown. x, A variable amino acid residue.

Screening of the PC12w cDNA library

Duplicate nitrocellulose filter lifts (Genescreen, New England Nuclear) were taken for each pool of the PC12w cDNA library. They were treated with 0.5 M NaOH/1.5 M NaCl for 5 min and with 1.5 M NaCl/0.5 M Tris/HCl (pH 7.5) for 7 min successively. After baking at 80 °C for 1 h, the filters were hybridized with 1×10^6 c.p.m./ml of the labelled probe in 50% (v/v) formamide/6 × SSC (1 × SSC: 0.15 M NaCl/0.015 M sodium citrate)/0.1 M sodium phosphate (pH 7.0)/1 % (w/v) SDS/2.5 × Denhardt's solution at 42 °C for 20 h. Washing was performed twice with 2 × SSC/0.1% (w/v) SDS for 30 min at 55 °C. X-ray films (X-OMAT, Kodak) were exposed to the filters with intensifying screens (Lightning plus, Kodak) at −70 °C for 16 h. Positive clones were screened repetitively in the same way until singled out. The phage DNA was prepared by the plate lysate method using a DE52 anion exchanger (Whatman). The insert was excised out by digestion with *Bam*HI and was integrated into pBluescript KS(+).

DNA preparation and sequencing

DNA preparation was carried out with Qiagen Mini and Midi kits unless stated otherwise. DNA (3–5 μg) was denatured in 10 μl of 0.2 M NaOH/0.2 mM EDTA for 30 min at 37 °C and then subjected to the DNA sequencing based on the dideoxynucleotide chain-termination method [12] utilizing 7-deaza-dideoxynucleotides (Sequenase, United States Biochemical, Cleveland, OH, U.S.A.). Plasmids harboring truncated inserts of varying lengths were prepared by the Erase-a-Base System (Promega) in both directions. All of these plasmids were sequenced.

Expression of rat PTPLP and membrane preparation of transfectants

The insert of rat PTPLP was blunted with T4 DNA polymerase, ligated with *Bst*XI adapters (Invitrogen) and integrated into an expression vector, pCDNAI (Invitrogen). The direction of the insert in the plasmid was confirmed by restriction mapping. COS-7 cells were transfected with the plasmid (5 μg/1×10^6 cells) in 140 mM KCl/3 mM NaCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4/4 mM $MgCl_2$ by electroporation (0.15 kV, 250 μF) in a Gene Pulser (Bio-Rad). After 48 h of culturing, the cells were washed with PBS (5 ml/plate) and then were homogenized at 4 °C in 5 mM EDTA/0.2 mM PMSF/5 μg/ml pepstatin/5 μg/ml leupeptin/5 mM Tris/HCl (pH 7.1). After centrifugation, which was performed for 15 min at 4 °C and 100000 *g*, precipitated plasma membranes were washed with the same buffer twice. They were suspended in 100 mM NaCl/1 mM $MgCl_2$/50 mM Tris/HCl (pH 7.2). Protein concentration was determined with a Bio-Rad protein assay kit.

Phosphatase activity

Phosphatase activity was measured according to Streuli et al. [8]. Raytide (60 μg) was ^{32}P-phosphorylated with pp60$^{c\text{-}src}$ at 37 °C for 16 h in 180 μl of 0.1 mM EDTA/0.015% (w/v) Brij 35/10 mM $MgCl_2$/0.03 % (w/v) BSA/0.07% (v/v) 2-mercaptoethanol/50 μM [γ-^{32}P]ATP (0.5 MBq/ml)/50 mM Hepes/NaOH (pH 7.5). The reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid/20 mM NaH_2PO_4 and 100 μl of 10 mg/ml BSA. The precipitates thus formed were collected, dissolved in 0.2 ml of 0.2 M Tris/HCl (pH 7.2) and reprecipitated with 1 ml of 10% (w/v) trichloroacetic acid/20 mM NaH_2PO_4. This washing procedure was repeated again. ^{32}P-phosphorylated myelin basic protein (MBP) was prepared as well in the same way. Labelled substrate and plasma membranes (0.3–3.0 μg) were incubated in 100 μl of 5 mM EDTA/10 mM dithiothreitol/25 mM Hepes/NaOH (pH 7.4) at 37 °C for 30 min. The reaction was stopped by addition of 900 μl of 4% (w/v) activated charcoal/2 mM NaH_2PO_4/90 mM sodium pyrophosphate/0.9 M HCl. After centrifugation, which was performed for 15 min at 4 °C and 1000 *g*, 0.5 ml of the supernatant was mixed with 10 ml of biodegradable counting scintillant (BCS; Amersham) and its radioactivity was counted.

RESULTS

Cloning and structure of rat PTPLP

Two sets of degenerate primers were designed for PCR to obtain a probe for the screening of a PTPase. As shown in Figure 1, they were based on the highly conserved amino acid sequences of PTPases and additionally contain cleavage sites for either *Bam*HI or *Eco*RI. The combination 2 (Figure 1) was employed in a cloning study of the *pyp*1 gene of *Schizosaccharomyces pombe* [13] but did not give good results in the present study. The combination 1 generated PCR products of ~ 200 bp, which were then subcloned into pBluescript KS(+). Six clones were sequenced and only one of them proved to encode a partial sequence of a protein whose amino acid sequence is 35.3% identical with that of human LAR. Using this probe we screened ~ 100000 clones of our PC12w cDNA library and selected three clones. These clones have inserts of different sizes, but gave

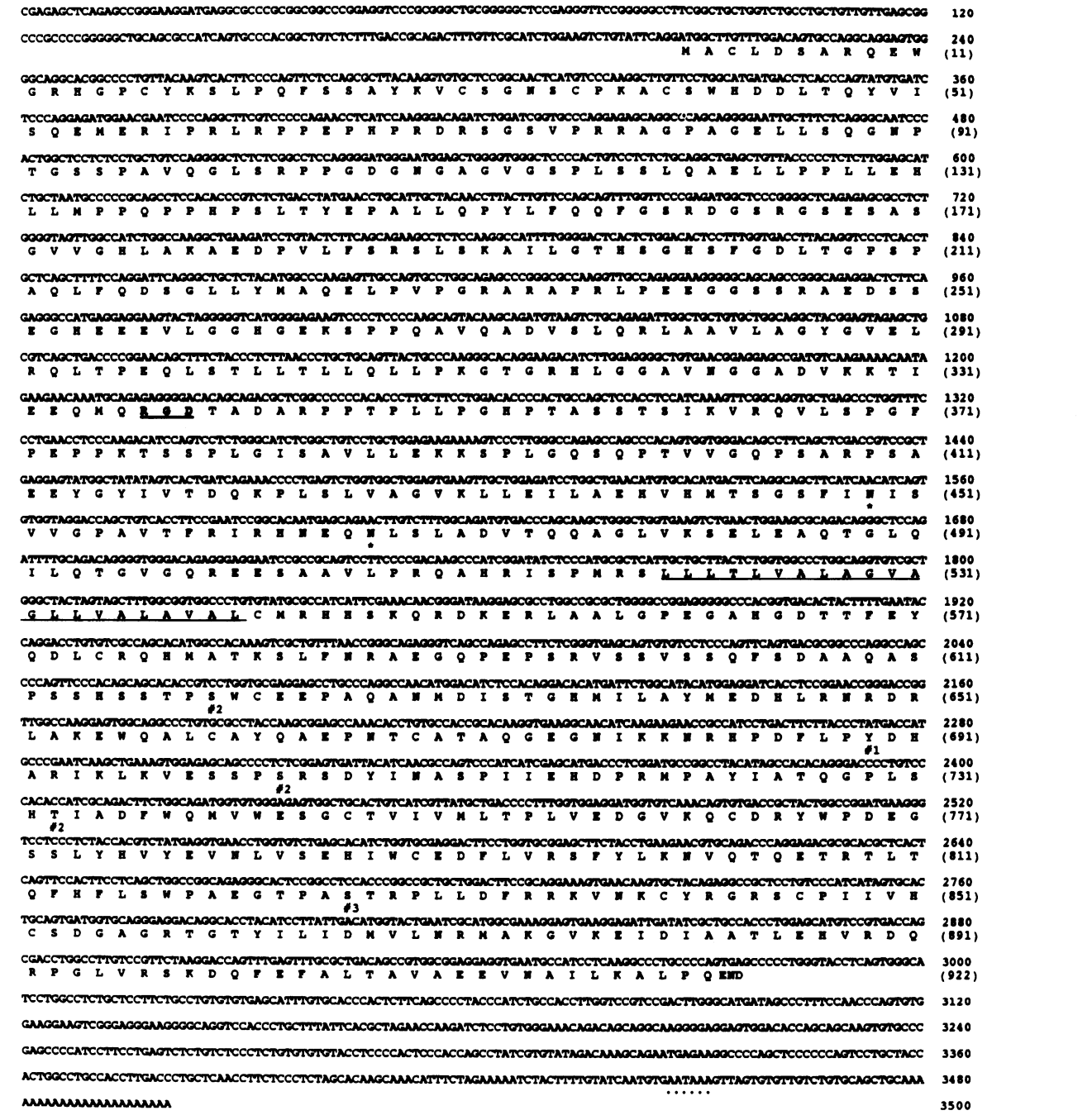

Figure 2 Amino acid and DNA sequences of rat PTPLP

The RGD motif and a membrane-spanning domain are underlined with solid lines. Potential N-glycosylation sites are indicated by asterisks. Potential sites of phosphorylation by tyrosine kinase, casein kinase II and protein kinase C are shown by #1, #2 and #3 respectively.

similar patterns when digested with *Kpn*I. We finally selected and sequenced a clone of the longest insert.

The clone which we subcloned as pGEM-PTPLP was carefully sequenced in both directions and proved to comprise 3500 bp, as shown in Figure 2. It has a 2766 nm open reading frame preceded by CAGG (positions 205–208), which is one of sequences frequently located before the initiation site of protein translation [14], and encodes a 922-amino-acid protein. Because a poly-adenylation signal (AATAAA) is located at positions 3447–3451, the poly(A) sequence at positions 3478–3500 appeared to be post-transcriptionally added.

Kyte–Doolittle hydropathy analysis [15] revealed that PTPLP has a single membrane-spanning domain at amino acid residues 520–541. Unlike usual PTPases, PTPLP contains only one PTPase-related domain. Other notable features of PTPLP are two potential N-glycosylation sites at residues 449 and 467 and one Arg-Gly-Asp (RGD) sequence at residues 337–339 in its extracellular domain. Moreover, it should be noted that PTPLP

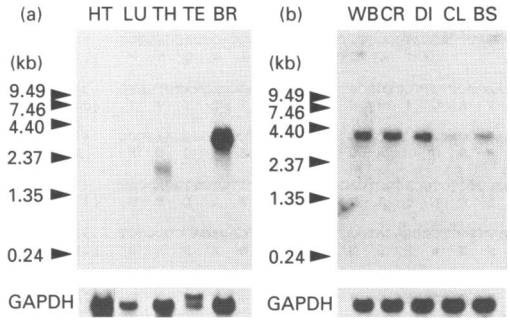

Figure 3 Northern blot analyses of rat PTPLP in various tissues (a) and in discrete regions of the brain (b)

Each lane contains 2 μg of poly(A)$^+$ RNA of rat tissues (male Sprague–Dawley rats): (**a**) samples of heart (HT), lung (LU), thymus (TH), testis (TE) and brain (BR); (**b**) samples of whole brain (WB), cerebral cortex (CR), diencephalon (DE), cerebellum (CL) and brain stem (BS). Molecular-mass standards (kb) are indicated on the left. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control probe.

contains potential phosphorylation sites for protein kinase C, casein kinase II and tyrosine kinase in its cytoplasmic domain.

Tissue distribution of PTPLP

Figure 3 shows the results of Northern-blot analyses of PTPLP. The protein was detected as a 4.0 kb transcript in the brain, but not in other tissues (Figure 3a). It is interesting that a short transcript of ~ 1.4 kb was detected in the thymus, although it is not clear whether or not it is an alternatively spliced variant. Moreover, we examined the expression levels of PTPLP in discrete regions of the brain. As shown in Figure 3(b), it was detected intensely in the cerebral cortex and in the diencephalon, mainly comprising hypothalamus and thalamus, but it was less and hardly detectable in the brain stem and in the cerebellum respectively.

Effects of PTPLP on phosphatase activity

Because PTPLP is a membrane-associated protein, we examined the PTPase activity of the membrane fraction of the COS-7 cells which were transfected with pCDNAI-PTPLP. The commercially available peptides Raytide and MBP were used as substrate. As shown in Table 1, the PTPase activity of the transfectants (590 units/mg of protein) was 70.1% that of the control cells (842 units/mg of protein), when ^{32}P-phosphorylated Raytide was used as substrate (29.9% decrease). However, neither a stimulatory nor an inhibitory effect was observed when ^{32}P-phosphorylated MBP was employed.

DISCUSSION

The present study was conducted to identify a membrane-associated PTPase from PC12w cells. Because the PC12w cells are a substrain of PC12 cells, which were derived from pheochromocytoma of adrenal medullary chromaffin cells and are known to be differentiated into a sympathetic-neuron-like morphology by nerve growth factor [9], the PTPase that would be cloned from a PC12w cDNA library was expected to be relevant to neuronal function. The clone that we isolated encodes a protein of considerable sequence identity with one of the PTPase catalytic domains of human LAR, and it was demonstrated that it is expressed specifically in the brain.

Table 1 PTPase activity of COS-7 cells transfected with expression plasmids

PTPase activities of the plasma membranes of the COS-7 cells transfected with pCDNAI-PTPLP (PTPLP) or pCDNAI (control) were determined. Labelled substrate (67 nM) and plasma membranes of the COS-7 cells (0.3–3 μg) were incubated in 0.1 ml of 5 mM EDTA/10 mM dithiothreitol/25 mM Hepes/NaOH (pH 7.4) at 37 °C for 30 min. After addition of 0.9 ml of 4% (w/v) activated charcoal/2 mM NaH_2PO_4/90 mM sodium pyrophosphate/0.9 M HCl, the supernatant (0.5 ml) obtained by centrifugation was mixed with 10 ml of BCS and its radioactivity was counted. One unit of activity is the amount of PTPase needed to release 3.33% of the total phosphate/min [37]. Data are expressed as means ± S.E. (n given in parentheses).

	PTPase activity (units/mg of protein)	
Substrate	Control	PTPLP
Raytide ($n = 5$)	842 ± 70	590 ± 64
MBP ($n = 4$)	552 ± 152	516 ± 80

Numerous PTPases have been cloned from a variety of sources [16]. Representative PTPases reported to be expressed in the brain are RPTPα [17], RPTPζ [18], RPTPγ [19], PTPσ [20] and so on. Among them, RPTPζ is restricted to specific regions, including Purkinje cells of the cerebellum. The present study has demonstrated that PTPLP is exclusively expressed in cerebral cortex, diencephalon and brain stem. Unlike other PTPases, it is expressed in a much lower amount in the cerebellum. PTPLP might play physiological roles distinct from those of the other PTPases in the brain.

Usually, PTPases possess two tandem catalytic domains. An intensive study of the roles of these two domains in the manifestation of the catalytic activity revealed that the second PTPase domain is important for substrate specificity [8]. Exceptionally, HPTPβ is known to possess only one catalytic domain [21]. In contrast with HPTPβ, which was reported to dephosphorylate Raytide as well as MBP [21], PTPLP appeared to slightly inhibit the PTPase activity of the plasma membranes of COS-7 cells when Raytide was used as substrate. It is likely that the overexpressed PTPLP affected the catalytic activity of the native PTPases of the COS-7 cells. If PTPLP had been produced in alkali-phosphatase-deficient bacteria and its PTPase activity had been assessed, such an inhibitory feature would not have been observed. Because the approach used was based on overexpression in mammalian cells, we could find that the PTPLP suppresses other PTPase activities. It should be noted that PTPLP has aspartate in place of alanine in the functionally important sequence motif [I/V]HCXAGXXR[S/T]G, as shown in Figure 4. Because the sequence of the PTPLP was determined in both directions using 7-deaza-dideoxynucleotides as terminators of DNA polymerase reaction, this mutation is not an error in DNA sequencing. Interestingly, Streuli et al. [8] demonstrated that the substitution of alanine with aspartate in this highlighted position led to complete elimination of the catalytic activity of LAR. Thus it is not surprising that PTPLP did not stimulate phosphatase activity in an assay system which was the same as the one that they employed.

It is interesting to speculate how PTPLP potentially could inhibit other PTPases. Its PTPase-like domain is anchored close to the plasma membrane, as is the first PTPase domain of other PTPases, which is thought to be functionally critical, unlike the second one, which is located more distantly from their transmembrane domain. Thus the cytoplasmic portion of PTPLP could interfere with the interaction of other PTPases with their substrate at their catalytic site. In the hypothesis proposed by Pot and Dixon [22], the catalytic activity of PTPase is manifested

Consensus sequence		V H C X **A** G X X R T G (I) (S)
PTPLP	[850–860]	- - - S **D** - A G - - -
LAR	[1520–1530]	- - - S - - V G - - -
LAR (inactively mutated)		- - - S **D** - V G - - -
HPTPε	[613–623]	- - - S - - A G - - -

Figure 4 Amino acid sequences of PTPLP and other representative PTPases at their PTPase sequence motif

The amino acid sequences of the consensus sequence [6], LAR and its inactive mutant [8], and HPTPε [21] are derived from the references indicated. The positions of the sequence motif are shown in brackets. The residue where deleterious replacement is observed in PTPLP is emphasized in **bold**. Residues matched to the consensus are denoted by hyphens in order to facilitate comparison.

through the nucleophilic attack of the cysteinyl sulfhydryl group towards phosphorylated tyrosine residues. The apparent slight reduction of PTPase activity by overexpression of PTPLP might be attributable to competitive inhibition by the functionally defected PTPase domain of PTPLP in the process of the nucleophilic attack of PTPases, but it is still uncertain.

We should be mindful that such an inhibitory profile of PTPLP does not necessarily represent its real function, because the interaction of PTPLP with its as-yet-unidentified extracellular ligand might be required to activate itself functionally, and such an interaction might result in activation or more evident inhibition of the PTPase activity. Indeed, the identities of the physiological ligands for receptor-type PTPases have been a very intriguing issue. The only example of such a ligand is CD45, which is a PTPase activated by the interaction with the T-cell receptor [23–25]. Most recently, it was demonstrated that neuronal cell adhesion molecule (N-CAM) and another extracellular matrix protein, tenascin, bind to chondroitin sulphate proteoglycan-type PTPases [26,27]. It was also reported that RPTPμ mediates cell–cell adhesion in SF9 cells [28,29]. In this respect, it is interesting that Arg-Gly-Asp, which is required for fibronectin to interact with integrin [30,31], is located in the extracellular domain of PTPLP. However, we cannot rule out that PTPLP could dephosphorylate other as-yet-unidentified substrates specifically, as the growth-factor-inducible immediate-early gene *3CH134*, which is known to be a PTPase, dephosphorylates mitogen-activated protein kinase specifically [32]. To understand fully the physiological function of PTPLP, identification of its own ligand and substrate(s) will be necessary.

In the course of this study, cloning studies on IA-2 [33] and ICA512 [34] were reported by two separate groups. IA-2 was isolated from a human insulinoma-derived subtraction library and ICA-512 was identified in human diabetic sera. ICA512 is considered to be a truncated form of IA-2, which is 79.3% identical with PTPLP in the amino acid sequence. Gebbink et al. [35] reported that human RPTPμ shares 98.7% sequence identity with its mouse homologue. Human LAR also shares extremely high similarity with rat LAR (as much as 94.9% identity in the amino acid sequence) [36]. Thus another protein much more closely related to rat PTPLP than to IA-2 might be expressed in human brain.

In conclusion, we have demonstrated that a protein distantly related to PTPases exists in PC12w cells and is exclusively distributed in the brain, particularly in cerebral cortex and diencephalon. Although its biological function is not yet fully understood, its inhibitory nature on other PTPases has been suggested in the present study.

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