Identification of a receptor-type protein tyrosine phosphatase expressed in postmitotic maturing neurons: its structure and expression in the central nervous system

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We have isolated a rat cDNA encoding a receptor-type protein-tyrosine-phosphatase (RTP) expressed in brain and kidney (RPTP-BK) and characterized its expression in the developing central nervous system. RPTP-BK has seven fibronectin type III-like repeats in the extracellular region and a unique catalytic phosphatase domain in the cytoplasmic region. Bacterial expression of its phosphatase domain showed that the dephosphorylation of phosphotyrosine residues was mediated by the cytoplasmic catalytic domain. Sequence comparison revealed that RPTP-BK is homologous with GLEPP1, a rabbit PTP expressed in renal glomerular epithelia, and has the same phosphatase domain as murine PTPβ expressed in macrophages. RPTP-BK has also significant homology with Drosophila.

INTRODUCTION

Reversible phosphorylation of tyrosine residues in proteins is responsible for various vital processes such as signal transduction, cell growth and homeostasis in many organisms [1]. Much effort has been focused on the understanding of these events at molecular levels. Protein phosphorylation is biochemically mediated by the two opposing dynamic activities of protein kinases and phosphatases. Protein phosphatases are a diversified group of enzymes that are categorized into two major families according to their substrate specificities: serine/threonine phosphatases and protein tyrosine phosphatases (PTPs, protein tyrosine phosphate phosphohydrolase, EC 3.1.3.48); there is also another group designated as dual-specificity phosphatases that are structurally related to PTPs but can dephosphorylate all the three phosphorylated residues.

Among protein phosphatases, PTP is the most numerous and ramified family, harbouring about 50 molecules currently identified. These PTPs are further divided into two main subgroups: the low-molecular-mass, cytoplasmic, PTPs containing a single catalytic domain, and the high-molecular-mass, membrane-spanning, receptor-type PTPs (RPTPs), many of which contain two tandem repeats of intracellular catalytic domains [2]. The catalytic domain consists of about 250 amino acid residues with several consensus sequences, allowing the reverse genetic approach to search for PTPs expressed in any tissues with reverse transcriptase–PCR (RT–PCR). The receptor-type PTP is characterized by its large extracellular motifs including immunoglobulin-like repeats and fibronectin type III (FN-III)-like repeats, which suggest ligand-binding activity. However, sequence similarities of the extracellular domain to neural cell adhesion molecules such as N-CAM, L1 and fasciclin II led us to speculate that homophilic or heterophilic cell-to-cell interactions rather than soluble ligands trigger the phosphatase activity as shown in PTPα and PTPζ [3,4].

In the nervous system, the regulation of protein functions through tyrosine phosphorylation catalysed by receptor tyrosine kinases is known to be crucial to the developmental process [5]. The function of these receptor tyrosine kinases has been extensively studied in Drosophila with several mutants [6]. However, the biological significance of PTP in the developing brain is not well understood. In a previous study we searched for PTPs expressed in rat embryonic brain by the RT–PCR method and detected the expression of several PTPs, including a novel PTP [7]. In the present study, we molecularly cloned this novel PTP from a rat embryonic brain cDNA library and characterized its structure and expression in the rat nervous system. This cDNA, designated as RPTP-BK because of its expression in the brain and kidney, encodes an RPTP and is homologous with rabbit GLEPP1 expressed in glomerular epithelia of kidney [8], and with murine PTPβ expressed in haematopoietic cells [9]. RNA blot analysis and hybridization in situ revealed that the

Abbreviations used: FN-III, fibronectin type III; LAR, leucocyte-antigen-related; MBP, maltose-binding protein; PTP, protein tyrosine phosphatase; RPTP, receptor-type protein tyrosine phosphatase; RT–PCR, reverse transcriptase–PCR.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U28938.
expression in the brain is developmentally regulated in the postmitotic maturing neuronal cells.

**MATERIALS AND METHODS**

**Isolation of rat RPTP-BK cDNA**

A rat embryonic brain cDNA library [10] was screened by the PCR fragment of PTPD28 [7]. One of the positive clones harbouring the largest insert was subcloned, and the reaction for sequencing was done with PCR by using SequiTherm polymerase and fluorescein isothiocyanate-labelled primers in accordance with the manufacturer’s instructions (Epitencire Technologies, Madison, WI, U.S.A.). The nucleotide sequence in both strands was determined with a DSQ-1 autosquencer (Shimazu, Kyoto, Japan). Multiple alignments for PTP genes were adjusted by the Clustal method in Lasergene software packages (DNASTAR, London, U.K.).

**Southern blot analysis**

Genomic DNA (10 µg) from Wistar rat liver was digested by several restriction enzymes. Digested DNA species were subjected to agarose-gel electrophoresis and were transferred to a nylon membrane. The 3.9 kb Xhol/Xbal fragment from RPTP-BK labelled with [32P]dCTP was used as a probe. The hybridization was performed in a solution containing 6 x SSPE [where SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 2 x Denhardt’s solution, 0.5 % SDS and, 100 µg/ml salmon sperm DNA at 65 °C for 12 h. The filter was washed in a solution of 0.2 x SSPE/0.1% SDS at 65 °C. The washed filter was exposed to Fuji X-ray film RX.

For zooblot analysis, the filter containing EcoRI-digested genomic DNAs from various species was purchased from BIOS Laboratory (New Haven, CT, U.S.A.). The 2.5 kb EcoRI/EcoRV fragment from RPTP-BK was used as a probe. Hybridization was performed as described above in the presence of 10 % (w/v) dextran sulphate. The filter was washed in a solution of 1 x SSPE/0.1% SDS at 65 °C and exposed to X-ray film.

**RNA preparation and RNA blot analysis**

Total RNA was extracted from rat tissues and cultured cells by the acid guanidinium thiocyanate/phenol/chloroform method [11]. The isolation of renal glomeruli was performed as described [12]. Messenger RNA species were prepared from rat embryonal, neonatal and adult brains with an RNA preparation kit (Pharmacia, Uppsala, Sweden). Isolated RNA (10 µg) or mRNA species (1 µg) were subjected to electrophoresis in a 1.2 % agarose/10 % formaldehyde gel, and blotted on a nylon membrane. The 3.9 kb Xhol/Xbal fragment labelled with [32P]dCTP was used as a probe. Hybridization was performed in a buffer containing 50 % (v/v) formamide, 5 x SSPE, 0.1% SDS, 5 x Denhardt’s solution and 100 µg/ml salmon sperm DNA at 42 °C for 16 h. The blots were washed at 50 °C in a solution of 0.2 x SSC/0.1% SDS (where SSC is 0.15 M NaCl/0.015 M sodium citrate) and exposed to X-ray film.

**Preparation of maltose-binding protein (MBP)–PTP fusion protein**

A fragment (nt 2830–3720) corresponding to the catalytic domain of RPTP-BK was subcloned into pMAL-c2 vector (New England Biolabs, Beverly, MA, U.S.A.). MBP–PTP and MBP–βgalactosidase fusion protein (as a control) were prepared in accordance with the manufacturer’s protocol (New England Biolabs). Briefly, JM104 cells harbouring the plasmids were inoculated into Luria-Bertani medium at 1:10 dilution and grown at 37 °C for 2 h, and then 0.3 mM isopropyl β-d-thiogalactoside was added. After the cells had been further propagated for 2 h they were suspended in a solution of 33 mM Tris/HCl (pH 7.5)/2.5 mM EDTA/10 mM 2-mercaptoethanol/1 mg/ml lysozyme/1 % (w/v) Triton X-100/10 µg/ml aprotinin/10 µg/ml leupeptin/1 mM PMSF, incubated at room temperature for 10 min and then sonicated on ice. The lysate was obtained by centrifugation at 10000 g for 10 min. Protein concentration was determined by the method of Bradford [13]. The aliquots were subjected to SDS/PAGE to confirm that they migrated appropriately for their expected molecular masses.

**Assay for phosphatase activity**

Phosphatase activity was assayed by dephosphorylating the synthetic peptide, Raytide (Oncogene Science, Uniondale, NY, U.S.A.) as described [14]. The peptide was labelled with [γ-32P]ATP by p60 ciso-Oncogene Science) at 37 °C for 12 h. Reaction mixtures with [γ-32P]-Raytide (2 x 106 c.p.m.) and bacterial lysates were incubated in 25 mM Hepes (pH 7.3)/5 mM EDTA/10 mM dithiothreitol at 37 °C. The reaction was terminated with acidic charcoal mixture [0.9 M HCl/90 mM sodium pyrophosphate/2 mM NaHPO4/4 % (v/v) Norit A]. After centrifugation the amount of radioactivity in the supernatant was measured and the phosphatase activity (released radioactivity) was expressed as a percentage of the total radioactivity used.

**Hybridization in situ**

Rat neonatal brain and kidney were fixed in 4 % (w/v) paraformaldehyde in PBS at 4 °C overnight, dehydrated with ethanol and embedded in paraffin. Serial sections (5 µm) were cut and mounted on poly-l-lysine-coated slides. After the removal of wax, sections were fixed in 4 % (w/v) paraformaldehyde, treated with 0.25 % (v/v) acetic anhydride in 0.1 M triethanolamine and dehydrated again. As anti-sense probe pGEM7Z plasmid (Promega, Madison, WI) harbouring an 808 bp PurI fragment (nt 2012–2819) of RPTP-BK cDNA was linearized and transcribed by SP6 polymerase with [γ-32S-UTP (more than 37 Tbq/ mmol; Amersham, Little Chalfont, Bucks, U.K.). Sense probe was synthesized by T7 polymerase after linearization. Hybridizations with 102 c.p.m./ml radioactively labelled probe were performed at 50 °C for 16 h in 50 % (v/v) deionized formamide/10 mM Tris/HCl (pH 7.6)/1 mM EDTA/600 mM NaCl/0.25 % SDS/1 x Denhardt’s solution/10 % dextran sulphate/10 mM dithiothreitol/200 µg/ml Escherichia coli tRNA. Samples were then treated with 12.5 µg/ml RNase A at 37 °C for 30 min. Washing was performed in a solution of 0.1 x SSC at 50 °C and slides were dehydrated with ethanol. Autoradiography was performed with Kodak NTB-3 emulsion diluted 1:1 with 2 % (v/v) glycerol in distilled water. Slides were developed with Kodak D-19 and fixed with FujiFix. Sections were counterstained with haematoxylin/eosin or Toluidine Blue.

**RESULTS**

**Molecular cloning of RPTP-BK cDNA**

We detected a novel PTP cDNA among the amplified genes for PTP from rat embryonic brain with the RT–PCR technique using degenerate primers corresponding to consensus motifs of the phosphatase domain [7]. To characterize the primary structure of this PTP molecule, we isolated a full-length cDNA clone from a rat embryonic brain cDNA library and designated it as
Cloning of a tyrosine phosphatase and its expression in the nervous system

Figure 1 Nucleotide and deduced amino acid sequences of rat RPTP-BK

The amino acid sequence is shown below the nucleotide sequence. Nucleotide numbering begins with the first nucleotide of the cDNA clone and the amino acid sequence begins with the leader sequence.

RPTP-BK (receptor-type PTP expressed in brain and kidney; see Figure 8).

The nucleotide sequence of 4871 bp RPTP-BK cDNA was determined and an open reading frame predicted the polypeptide of 1215 amino acid residues with a molecular mass of 137939 Da and an isoelectric point of 6.08 (Figure 1). The sequence around a putative initiator methionine codon is consistent with the Kozak consensus sequence [15]; however, we failed to detect polyadenylation signals in the 3' untranslated region. The nucleotide and deduced amino acid sequences were compared with the sequences in the GenBank (Release 91.0) and SWISS-PROT (Release 32.0) databases respectively. The survey suggested that RPTP-BK is a rat homologue of GLEPP1, a rabbit or a human PTP expressed predominantly in glomerular epithelia of kidney [8,16]. The homology between RPTP-BK and rabbit GLEPP1 or human GLEPP1 was 81.4% and 82.5% respectively at the nucleotide level, and 89.0% and 90.7% respectively at the amino acid level. The sequence alignment also showed that RPTP-BK has an additional 28 amino acid residues (877–904) in the juxtamembrane cytoplasmic region compared with GLEPP1.

Characterization of primary structure of RPTP-BK

The deduced amino acid sequence of RPTP-BK showed a putative signal peptide sequence (residues 1–29), a single hydrophobic amino acid stretch compatible with a transmembrane portion (residues 819–843) and a unique catalytic domain (residues 917–1192) (Figure 2). Thus RPTP-BK is classified as type III RPTP [17], including HPTPβ [18] and DPTP10D [19,20], which is expressed specifically in a subset of developing axons in the Drosophila central nervous system. In the extracellular domain there are seven repeats of FN-III-like domain (Figures 2 and 3), whereas HPTPβ and DPTP10D have 16 and 12 FN-III-like repeats respectively.

We compared the primary structure of the catalytic domain of RPTP-BK with those of other RPTPs. As shown in Figure 4, the phosphatase domain of RPTP-BK is highly homologous with GLEPP1 (95% amino acid similarity) and to DPTP10D (48%). It also possesses about 35% homology with each domain of HPTPβ, leucocyte-antigen-related (LAR) [21], PTPµ [22] and CD45 [24]. Crystallographic studies on PTP1B showed that the C-terminal region of this domain forms the binding pocket for phosphotyrosine [25,26]. The residues at the base of the binding pockets were well conserved among RPTPs, from Trp to Pro and from His to Ile (Figure 4), although RPTP-BK showed Ala-Leu instead of the conserved Gly-Val.
Figure 3 Alignment of FN-III-like repeats in the extracellular domain of RPTP-BK

Seven FN-III-like repeats in RPTP-BK are aligned in accordance with the consensus sequence [30]. Amino acid residues matched with the consensus are indicated by filled boxes. Gaps have been introduced to maximize the alignment.

Figure 4 Alignment of phosphatase domains of RPTP-BK and other PTPs

Primary sequences of phosphatase domains from RPTP-BK, GLEPP1, DPTP10D, HPTPβ, LAR, PTPβ, PTPγ and CD45 were aligned by the Clustal method. Amino acid residues that showed at least four identical residues among PTP members are indicated by filled boxes. Gaps have been introduced to maximize the alignment. Asterisks denote the segments corresponding to the binding pocket for phosphotyrosine [25,26].

To analyse the molecular evolution of RPTP-BK-related phosphatases, we calculated the genetic distances on the aligned sequences of the catalytic domain and made a phylogenetic tree (Figure 5). The tree suggests that RPTP-BK has evolved from the same ancestor gene as have DPTP10D and HPTPβ, although the extracytoplasmic domains among these molecules are less similar than the cytoplasmic domains (results not shown). The structural divergence in the extracellular domain thus implies that a putative ancestral PTP gene underwent complex evolutionary processes. The deduced amino acid sequence suggests that RPTP-BK has 14 possible N-linked glycosylation sites in its extracellular domain and two sites in its cytoplasmic domain (Figure 2B). We also found two putative tyrosine kinase phosphorylation sites at Tyr67 and Tyr196, and a cAMP-dependent protein kinase phosphorylation site at Thr415. There are also 13 and 19 potential sites for phosphorylation by protein kinase C and casein kinase II respectively (results not shown).

RPTP-BK is well conserved in various animals

We performed a Southern blot analysis to examine the genomic organization of RPTP-BK in rat and detected a single ApeI fragment (10 kb) and two BglII fragments (15 and 7 kb) (Figure 6A). This implies that RPTP-BK gene is a single copy and there is at least one intron sequence within the RPTP-BK genomic locus. Because we observed multiple bands in DNA digested with other restriction enzymes, we needed a more detailed map to determine the whole genomic organization. In zooblot analysis, a rat RPTP-BK cDNA probe detected a unique
A recombinant phosphatase domain from RPTP-BK was produced in *E. coli* as a fusion protein with MBP. Bacterial lysates (10, 2 and 0.4 ng) were assayed for phosphatase activity against ^32^P-labelled Raytide peptide as a substrate. Bacterial lysate containing MBP–lacz was used as a control. The line for MBP–lacz runs along the x-axis. The activity (free phosphate released) is expressed as a percentage of the total radioactivity used.

**Figure 7** Phosphatase activity of RPTP-BK

**DISCUSSION**

In the present study we isolated a full-length RPTP-BK cDNA that encodes a type III RPTP with seven FN-III-like repeats in the extracellular region, a single transmembrane domain and one catalytic phosphatase domain in the cytoplasmic region. Thomas et al. [8] proposed eight repeats of the FN-III domain in GLEPP1, a rabbit homologue of RPTP-BK, but the first repeat in the most N-terminal portion seems to be aberrant and does not conform to the consensus. Genomic Southern blot analysis suggests that RPTP-BK is expressed in developing brain and renal glomeruli

Northern blot analysis showed that the hybridized band migrated at 5.6 kb, corresponding to the cDNA obtained, and revealed the restricted expression of RPTP-BK in brain and kidney (Figure 8). Transcription in brain is dynamic during the neonatal stage. We detected hardly any expression in embryos at day 16 post coitum, but significant expression in 1-day-old pups. The transcript decreased 14 days after birth, and was further down-regulated in adult brains but still detectable in a trace amount (Figure 8A). We also investigated expression in renal tissues and various renal tubular cell lines. RPTP-BK was specifically expressed in isolated glomeruli, but not in mesangial cells, glomerular endothelial cells or tubular cell lines such as LLCPK1, MDCK and MDBK (Figure 8B).

To analyse the spatial and temporal regulation of RPTP-BK we investigated the expression of RPTP-BK in rat neonatal brain and kidney by hybridization *in situ*. In the brains of 3-day-old pups, we observed the strongest expression in the olfactory bulb and cerebral cortex and definitive signals *in situ*, although less strong, in the thalamus, hippocampus, midbrain regions such as the superior colliculus, inferior colliculus and interpeduncular nucleus, and in the brainstem (Figure 8A). In the olfactory bulb the signals were specifically localized to the granular cell layer and the mitral cell layer but were hardly detected in precursor neurons of the subventricular zone (Figure 8B). This suggests that RPTP-BK mRNA is specifically transcribed in postmitotic maturing neurons. In addition to the expression in the olfactory bulb, predominant expression of RPTP-BK was also localized in the olfactory tubercle (Figure 9A) and the piriform cortex (results not shown), suggesting that RPTP-BK is involved in the olfactory-rhinencephalon system. In the kidney, RPTP-BK expression was detected exclusively in the renal glomeruli of newborns (Figures 9C and 9D) and of adult rats (results not shown). The expression in the kidney is not developmentally regulated but is constitutive irrespective of the developmental stage.

**RPTP-BK is expressed in developing brain and renal glomeruli**

We prepared recombinant protein in which the catalytic domain (Asp^65^ to Ser^106^) from RPTP-BK was fused to MBP, to examine the phosphatase activity of RPTP-BK. As shown in Figure 7, MBP–PTP, harbouthing the catalytic domain of RPTP-BK, showed phosphatase activity for ^32^P-Tyr-Raytide in a dosedependent manner, whereas the control, MBP–lacz, failed to show phosphatase activity. In the assay, 10 ng of bacterial lysate containing the recombinant catalytic domain released 90% of the incorporated phosphate from the labelled Raytide peptide in 10 min and the reaction reached a plateau. In contrast, 0.4 ng of bacterial lysate showed a time-dependent increase in released phosphate up to 30 min. These results indicate that RPTP-BK has an intrinsic enzyme activity of tyrosine phosphatase in its cytoplasmic region, like other receptor-type PTPs.

**Phosphatase activity of recombinant RPTP-BK**

**Figure 8** Expression of RPTP-BK in developing brain and renal glomeruli

(A) mRNA (1 µg) from rat embryonal (E16), juvenile (P1 and P14) and adult brains was separated by formaldehyde gel electrophoresis, blotted on a nylon membrane and hybridized with a RPTP-BK cDNA probe. The same blot was rehybridized with a probe of glyceraldehyde-3-phosphate dehydrogenase cDNA as a control. (B) A blot carrying 10 µg of total RNA from rat liver, kidney, isolated glomeruli, cultured mesangial cells, cultured glomerular endothelial cells, LLCPK1, MDCK and MDBK was hybridized with an RPTP-BK cDNA probe. The same blot was rehybridized with a probe of rat EF-1α cDNA as a control.
Thus the 47 kDa PTPφ amino acid stretch of RPTP-BK, which is not found in GLEPP1.

Because three isoforms of PTPφ, including a cytoplasmic isoform, were generated by an alternative use of «untranslated sequence» exons [27]. Thus the underlying molecular mechanism of generating isoforms might not be unprecedented in PTP genes.

In the present study we examined the temporal and spatial expression of RPTP-BK in the developing nervous system and demonstrated that the transcript was localized in neurons of the olfactory bulb, cerebral cortex and other regions at the neonatal stage. The expression in the cerebral cortex of neonatal brain was down-regulated thereafter, but it remained unchanged during and after the maturation of the olfactory bulb and allocortex. The constitutive expression was also observed in the kidney irrespective of developmental stage (results not shown).

Yang et al. [20] identified a Drosophila PTP, DPTP10D, and showed that it was selectively expressed in a subset of developing axons and pioneer neurons in the embryonic central nervous system. Because its temporal expression is correlated with the development of major axon tracts, Yang et al. speculated that it plays definitive role(s) in axon outgrowth and/or neuronal guidance. In our study the expression of RPTP-BK in the developing central nervous system coincided with the stage of neuronal axonogenesis [28]. It is interesting to note that RPTP-BK is dominantly expressed in the regions where neuronal axonogenesis takes place, but not in precursor neurons that failed to show axonogenesis, such as those in the subventricular...
zone. Because RPTP-BK is highly similar to DPTP10D, RPTP-BK might play pivotal role(s) in axon outgrowth and neuronal guidance in the mammalian central nervous system.

Recently, Desai et al. [29] characterized Drosophila mutants lacking PTPs DPTP69D and DPTP99A, and showed that they were required for motor axon guidance. They argued that these RPTPs might be involved in the defasciculation of bundled axons for proper pathfinding in the peripheral nervous system. It prompts us to speculate that the central nervous system has similar mechanisms for pathfinding in axonogenesis. Considering the biological significance of RPTP-BK, mutant mice lacking RPTP-BK would shed light on these issues in future.

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