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cDNA cloning of a cytosolic protein tyrosine phosphatase (RKPTP) from rat kidney

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Abstract A rat cDNA encoding a non-receptor type phosphotyrosine phosphatase (PTPase; EC 3.1.3.48) was identified. The 1608 bp cDNA contains a single open reading frame that predicts a 382 amino acid protein with M_r 44,438. The predicted protein has no apparent signal or transmembrane sequences, suggesting that it is a cytosolic protein. The C-terminal region has a PTPase catalytic domain that has 40–50% nucleic acid homology to other known PTPases. The N-terminal region has little amino acid sequence homology to any other known sequences. The recombinant protein of the cloned cDNA expressed in *Escherichia coli* was shown to possess PTPase activity using myelin basic protein, tyrosine phosphorylated by $p43^{v\text{-}abl}$ tyrosine kinase, as a substrate.

Key words: Protein tyrosine phosphatase; Rat kidney; Protein phosphorylation

1. Introduction

Protein phosphorylation on tyrosine residue is one of the main eukaryotic cell signaling mechanisms and plays a crucial role in cell proliferation and differentiation [1]. Protein tyrosine phosphorylation is transient and is reversibly regulated by the antagonistic effects of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). Like PTKs, PTPases are thought to play a role in the cell signaling pathway involved in proliferation and differentiation. Recent findings have shown the involvement of PTPases in density-dependent cell growth control in various types of cells [2,3]. Heterologous PTPase activities have been detected and characterized in both soluble and particulate fractions derived from a wide variety of cell types and tissues [4], suggesting the existence of a diverse PTPase gene family. The PTPases constitute a rapidly growing and diverse family of enzymes that exist as both transmembrane and cytoplasmically localized forms. Members of the PTPase gene family share a high degree of homology in PTPase domain (about 240 amino acids). We attempted to identify novel members of the PTPase gene family that could participate in growth regulation of the cell. In this paper, we present the identification of a widely expressed cytosolic PTPase (RKPTP) isolated from rat kidney.

2. Materials and methods

2.1. Materials

Restriction endonucleases and modifying enzymes were obtained from Takara Shuzo (Kyoto, Japan), Life Technology (Gaithersberg, MD), and Toyobo (Osaka, Japan). Recombinant *Thermus aquaticus* (*Taq*) polymerase was from Perkin-Elmer Cetus.

2.2. Polymerase chain reaction (PCR) cloning of PTPase-related sequences

The amplification of PTPase sequences using degenerate oligonucleotide primers for PTPase conserved regions has already been described [5]. PCR primer P3 [sense; 5′-T(G/C)(A/G)(A/G)(T/G/C)GA(T/C)TT(T/C)TGG(A/C)(G/A)(A/G/C)ATG-3′], and primer P4 [antisense; 5′-(A/G)CTCCTGC(A/G/C/T)CT(A/G)CA(A/G)TG(G/C)AC-3′] were synthesized based on the partial sequence of the peptides N/GDFWRM and VHCSAG. Single-stranded cDNAs were synthesized from rat kidney total RNA by random hexamer priming and MMLV reverse transcriptase. The PCR was performed as follows. The first and second cycles were for 1 min at 94°C (denaturing), 1 min at 37°C (annealing), 1 min at 72°C (extension). The following 28 cycles were carried out under the same conditions except for the annealing temperature (the 3rd and 4th cycles at 45°C and the 5th to the last cycles at 55°C). PCR products of expected size (about 350 bp) from rat kidney cDNA amplified by using primer pair P3 and P4 were gel-purified and subcloned into pUC119 and were sequenced.

2.3. Isolation of PTPase cDNA

A total of 5 rat cDNA libraries (4 from kidney, 1 from NRK cell) was constructed according to Gubler and Hoffman [6] in λgt10 and was screened with PCR clone p34-11 by the plaque hybridization procedure [7]. Approximately 5×10^6 plaques were screened. Inserts for positive clones were subcloned in pBluescript II SK(+) (Stratagene) after digestion with *Eco*RI. Sets of progressive deletions were produced by timed exonuclease III digestion, mung bean nuclease treatment and re-ligation [7]. Nucleotide sequencing was carried out by the dideoxy termination method using denatured plasmid DNA. Since the 5′ non-coding region of the RKPTP showed high (G+C) content, heat stable *Bca*BEST DNA polymerase (Takara Shuzo, Kyoto, Japan) was used to determine the nucleotide sequence of that region.

2.4. Bacterial fusion protein

The PTPase cDNA (nucleotides 154–1608) was cloned in-frame with glutathione-*S*-transferase (GST) in the pGEX-3X (Pharmacia) bacterial expression vector. DH5α bacteria transformed with pGEX-3X plasmid was grown in Luria broth to an absorbance at 600 nm of 0.5 and was induced with 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) for 3 h. Isolation of GST fusion protein was carried out using glutathione-Sepharose 4B (Pharmacia) as described [8].

2.5. Phosphatase assay

Bovine brain myelin basic protein (MBP) (Sigma) was labeled in its

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The nucleotide sequence reported in this paper will appear in the GSDB, DDBJ, EMBL, NCBI nucleotide sequence database under accession no. D38072.

Abbreviations: PTPase, protein tyrosine phosphatase; PTK, protein tyrosine kinase; PCR, polymerase chain reaction; GST, glutathione-*S*-transferase; MBP, myelin basic protein.

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tyrosine residues using [γ-^{32}P]ATP and p43$^{v\text{-}abl}$ tyrosine kinase (Oncogene Science, Manhasset, NY) essentially as described by the suppliers except that the reaction mixture was incubated at 37°C for 16 h. The labeling was terminated by adding 0.5 ml of 20% (w/v) activated charcoal (500 μl). The activated charcoal absorbed ^{32}P-labeled myelin basic protein but could not bind the released phosphate. After centrifugation in a microfuge, the amount of radioactivity in the supernatant was measured.

2.6. Northern and Southern blot analyses

Total RNA was extracted from adult rat tissues by acid guanidinium thiocyanate-phenol-chloroform method as described [9]. Fifteen μg of total RNA was electrophoresed in a formaldehyde/0.9% agarose gel, blotted to a nylon membrane filter, and hybridized to ^{32}P-labeled probe. Rat genomic DNA was isolated from rat liver as described [7]. For Northern and Southern blotting, p34-11 (nucleotides 483–836) was used as a probe. The final wash was in 0.1 × standard saline citrate/0.1% SDS at 65°C for 20 min.

3. Results and discussion

3.1. Amplification of PTPase-related sequences by PCR

We obtained 3 independent clones (P34-6, P34-7, P34-11) which have a conserved amino acid stretch characteristic of PTPases [10]. P34-6 was identical to rat LAR (leukocyte common antigen related protein) [11] and P34-7 turned out to be a rat counterpart of mouse LRP (leukocyte common antigen-related phosphatase). The full-length cDNA for the latter one has been cloned and reported already [5]. Since P34-11 had at most 40–50% nucleotide sequence homology to the other known molecules, this clone was considered to be derived from a novel member of the PTPase gene family. Then, we proceeded with the full-length cDNA cloning of P34-11.

3.2. Isolation of cDNA for RKPTP from rat kidney cDNA library

A rat kidney cDNA library constructed in λgt10 was screened using P34-11 as a probe. Subsequent screening of rat kidney libraries (5×10^6 recombinants) was carried out with various random hexamer-primed radiolabeled cDNA probes. We obtained several overlapping phage clones which were isolated and subcloned into plasmids, and sequenced. One open reading frame was identified with 382 predicted amino acids (Fig. 1), and this new molecule was designated RKPTP. The C-terminal portion of the RKPTP has little sequence similarity to the other known molecules in the GenBank database. The 5′ end of this clone was extremely (G+C) rich (81% in the region 5′ of the putative initiation codon). This feature might explain the relative rarity of the clones bearing this region of the RKPTP mRNA in any of the libraries screened. This type of element has been described by Kozak [12] as a characteristic feature of the mRNAs of a number of proteins that are involved in cell growth control, including many growth factor receptors.

3.3. Bacterial expression and PTPase activity

To confirm that RKPTP functions as a PTPase, the RKPTP cDNA was cloned into the bacterial expression vector,

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CCGGCCGGTGCCGCTGGCAGCCGCTAGCCGCCTCGCTGCTGGCGGTGGTAGGGTAGGGCGGTGCTCGGAGCGAGC   75
TGGGGAAGACGGAGCGGGCGCTGCGGCGCGCGGGCGGGCGGCGGGGGGGACGCGGGAGGATGGAGCAAGTGGAGA  150
                                                          M  E  Q  V  E  I    6
TTCTGAGGAGGTTCATCCAGAGGGTCCAGGCCATGAAAAGTCCGGACCACAATGGGGAGGACAACTTCGCCCGGG  225
  L  R  R  F  I  Q  R  V  Q  A  M  K  S  P  D  H  N  G  E  D  N  F  A  R  D   31
ACTTCATGCGATTGAGAAGATTGTCTACCAAATATAGAACAGAAAAGATTTATCCCACAGCCACTGGAGAAAAAG  300
  F  M  R  L  R  R  L  S  T  K  Y  R  T  E  K  I  Y  P  T  A  T  G  E  K  E   56
AAGAAAATGTTAAAAAGAACAGATATAAGGACATACTGCCATTTGATCACAGCCGAGTTAAGTTGACTTTGAAGA  375
  E  N  V  K  K  N  R  Y  K  D  I  L  P  F  D  H  S  R  V  K  L  T  L  K  T   81
CACCATCCCAAGATTCAGATTATATCAATGCAAATTTTATTAAGGGTGTATATGGGCCGAGAGCATACGTGGCAA  450
  P  S  Q  D  S  D  Y  I  N  A  N  F  I  K  G  V  Y  G  P  R  A  Y  V  A  T  106
CCCAAGGGCCTTTGGCGAATACAGTCATAGACTTCTGGAGGATGATATGGGAGTACAATGTGGTGATCATTGTAA  525
  Q  G  P  L  A  N  T  V  I  D  F  W  R  M  I  W  E  Y  N  V  V  I  I  V  M  131
TGGCCTGTCGTGAATTTGAGATGGGAAGGAAAAAGTGTGAGCGCTATTGGCCTTTGTATGGAGAAGATCCTATAA  600
  A  C  R  E  F  E  M  G  R  K  K  C  E  R  Y  W  P  L  Y  G  E  D  P  I  T  156
CATTTGCACCATTTAAAATTTCTTGTGAAAATGAACAAGCAAGAACAGACTACTTCATTCGAACACTTTTACTTG  675
  F  A  P  F  K  I  S  C  E  N  E  Q  A  R  T  D  Y  F  I  R  T  L  L  E  181
AATTTCAAAATGAATCCCGTCGACTCTATCAGTTTCATTACGTGAACTGGCCAGACCATGATGTCCCTTCGTCAT  750
  F  Q  N  E  S  R  R  L  Y  Q  F  H  Y  V  N  W  P  D  H  D  V  P  S  S  F  206
TCGATTCTATCCTGGACATGATAAGTCTAATGAGGAAGTACCAAGAGCACGAAGATGTGCCTATTTGTATCCATT  825
  D  S  I  L  D  M  I  S  L  M  R  K  Y  Q  E  H  E  D  V  P  I  C  I  H  C  231
GCAGTGCAGGCTGTGGACGGACAGGTGCTATTTGTGCCATCGACTACACGTGGAACTTACTGAAAGCAGGGAAAA  900
  S  A  G  C  G  R  T  G  A  I  C  A  I  D  Y  T  W  N  L  L  K  A  G  K  I  256
TTCCAGAGGAATTTAATGTATTTAATTTAATACAAGAAATGAGAACACAAAGGCACTCTGCAGTACAAACGAAGG  975
  P  E  E  F  N  V  F  N  L  I  Q  E  M  R  T  Q  R  H  S  A  V  Q  T  K  E  281
AGCAGTATGAACTCGTCCATAGAGCTATTGCCCAACTGTTTGAAAAACAGCTACAACTGTATGAAATCCACGGAG 1050
  Q  Y  E  L  V  H  R  A  I  A  Q  L  F  E  K  Q  L  Q  L  Y  E  I  H  G  A  306
CCCAGAAAATCACTGATGGTAATGAAATTAGCACTGGAAACATGGTCAGTTCCATTGATAGTGAAAAACAAGATT 1125
  Q  K  I  T  D  G  N  E  I  S  T  G  N  M  V  S  S  I  D  S  E  K  Q  D  S  331
CTCCTCCTCCAAAGCCACCACGGACTCGAAGTTGTCTTGTAGAGGGGGATGCCAAGGAAGAAATCCTCCAGCCAC 1200
  P  P  P  K  P  P  R  T  R  S  C  L  V  E  G  D  A  K  E  E  I  L  Q  P  P  356
CAGAGCCTCACCCGGTGCCGCCCATCCTGACACCGTCGCCTCCTTCAGCATTCCCAACGTTACACTGTGTGGCAA 1275
  E  P  H  P  V  P  P  I  L  T  P  S  P  P  S  A  F  P  T  L  H  C  V  A  R  381
GACAGTGACAGGTACCACCCAAAGCCAGTGCTGCACATGGCTTCCCCAGAGCAGCCCCCAACGGACCTCAACAGA 1350
  Q  *                                                                        382
AACTATGATAAGTCAGCGGACCTAATGGGGAGAAGCGAATCTGCTGTTGAGCACACAGATAAAAAGTTAGAACAA 1425
AATTTAAGTTTTGAGATTAAGAAAGTCCCTCTCCAAGAAGGGCCCAAAAGTTTTGATGGGAACACACTCTTGAAT 1500
AGGGGACATGCGATTAAAATTAAGTCTGCTTCATCTTCTGTAGTTGACAAAAGCTCTAAGCCACAGGAGTTAAGT 1575
TCAGGTGATCTAAAGGTTCACGATGTGTCTCAG                                            1608
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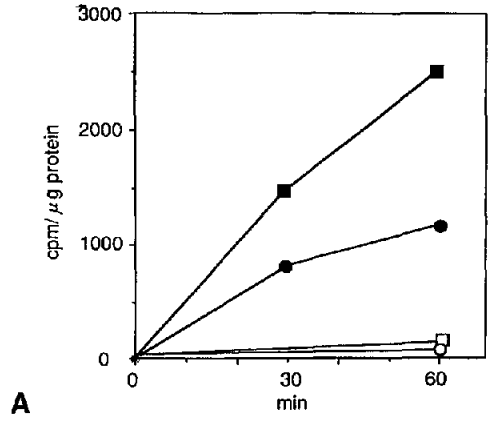
Fig. 1. Nucleic acid sequence and translated amino acid sequence of the RKPTP cDNA. The standard one letter code is used. In-frame termination codons are underlined.



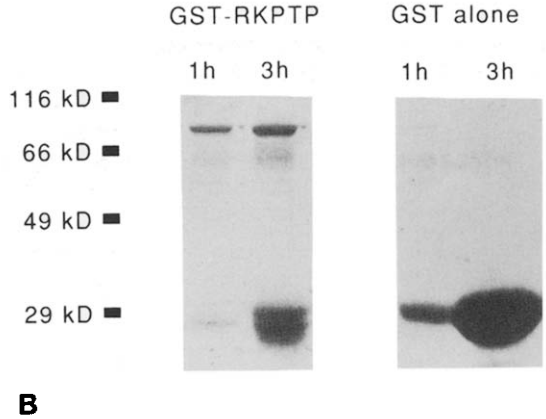


Fig. 2. (A) PTPase activity of RKPTP expressed in *E. coli*. The time-course is shown for dephosphorylation of [^{32}P]phosphotyrosine-containing myelin basic protein (2×10^5 cpm) incubated with 2 μg of supernatant protein or partially purified protein using glutathione-Sepharose 4B from DH5α cells transformed with either pGEX-RKPTP or pGEX. ●, GST-RKPTP lysate; ■, GST-RKPTP bead-purified; ○, GST alone lysate; □, GST alone bead-purified. (B) Coomassie blue staining of the SDS-polyacrylamide gel of recombinant GST-RKPTP or GST alone. Fusion proteins were partially purified from the supernatant protein from DH5α cells transformed with either pGEX-RKPTP or pGEX using glutathione-Sepharose 4B after the indicated time of induction by IPTG.

pGEX-3X, and the GST-RKPTP fusion protein was expressed in *E. coli*. Homogenates of the *E. coli* transformed with pGEX-RKPTP and the affinity-purified GST-RKPTP fusion protein were tested for PTPase activity as determined with [^{32}P]phosphotyrosine-containing MBP as a substrate. Both bacterial lysate and GST-RKPTP had PTPase activity, whereas little PTPase was demonstrated in the control bacterial lysate and GST alone (Fig. 2A). On SDS-PAGE, GST-RKPTP fusion protein was detected as a Coomassie blue staining band, the molecular weight of which was about 80 kDa (Fig. 2B).

3.4. Northern and Southern blot analyses

Northern blot analysis of RKPTP in rat tissues, including kidney, spleen, brain, liver, heart, and lung, is presented in Fig. 3. Ethidium bromide staining of 28 S and 18 S ribosomal RNA is also shown to judge the equality of the amount of RNA loaded per lane. We probed total RNA with P34-11. The major transcript was seen at 2.7 kb in all tissues tested. The strongest

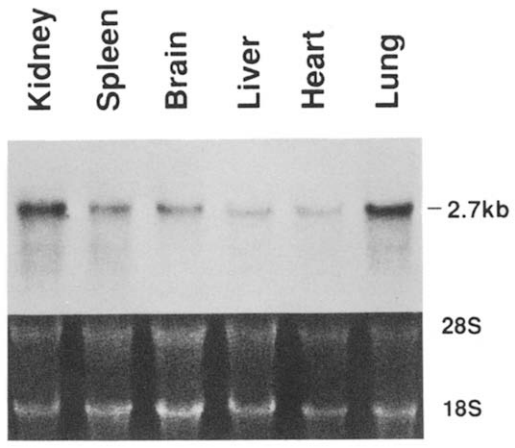


Fig. 3. Detection of RKPTP mRNA from various rat tissues by Northern blot analysis. Each lane was loaded with 15 μg total RNA. Ethidium bromide staining of the 28 S and 18 S ribosomal RNA is shown in the lower panel.

signal for RKPTP was found in kidney and lung. Hybridization of RKPTP cDNA to rat genomic DNA detected a single *Pst*I fragment (Fig. 4). There are two *Pst*I recognition sites in the cDNA, and as a probe we used a cDNA fragment (nucleotides 483–836) located between two *Pst*I sites on the cDNA. This result suggests that there is a single copy of the RKPTP gene per rat haploid genome.

3.5. Comparison of the core sequences of PTPase domains

The amino acid sequences of the core regions of 4 PTPases (3 from cytosolic PTPase, and 1 from transmembrane-type PTPase) are compared in Fig. 5. PTP1B is a prototype of cytosolic PTPase [13], and PTP1C was chosen because this was the first cloned SH2 domain containing PTPase [14]. From the transmembrane-type PTPase, rat LRP is shown. Only the domain I sequences of LRP are shown in this figure, because it is still controversial whether the domain II has catalytic activity. Within the core PTPase region, 47 amino acids are invariant, while many other amino acids are highly conserved. There

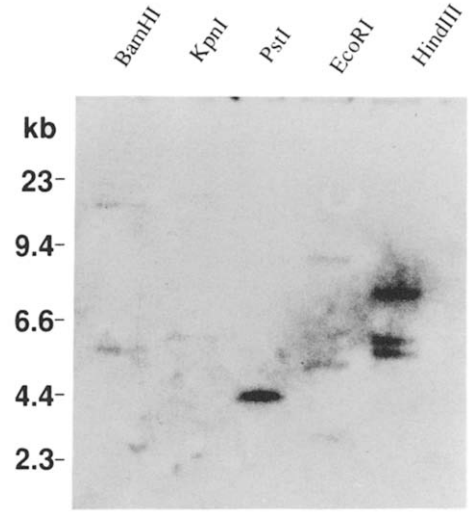


Fig. 4. Genomic Southern analysis of the RKPTP gene. Rat genomic DNAs were digested with the indicated restriction enzymes, separated and transferred to a nylon membrane filter.

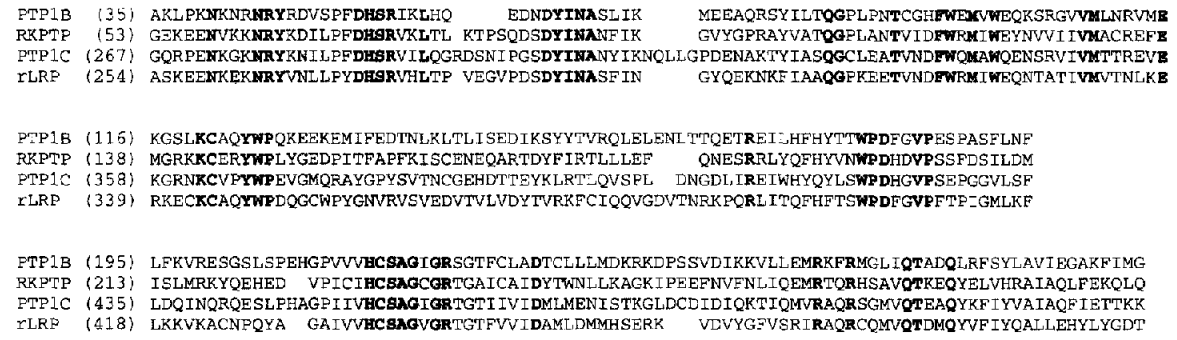


Fig. 5. Alignment of the core PTPase domain sequences. PTP1B and PTP1C are human sequences, whereas RKPTP and LRP are rat sequences. The positions where 4 PTPases share identical amino acids are in bold type.

are several clusters of conserved amino acids. The most notable cluster is the consensus sequence HCSAG(I/V)GR. Seven out of these 8 amino acids are conserved. This conserved stretch of sequence is important for PTPase activity, because it contains the cysteine residue that has been shown to be essential for PTPase enzyme activity [10]. It should be noted that there is a substitution in RKPTP at the (I/V) position of the consensus sequence to C, which is unique among the reported PTPases to date. The nucleotide sequences encoding the catalytic domain of RKPTP (nucleotides 306–980) was compared to the 5 cytosolic PTPases using the Maximum Matching program in Genetyx (version 8.0). The catalytic domain of HePTP [15] showed the highest homology of 53.7%, followed by LC-PTP [16] (52.8%). MPTP [17], PTP-S [18], and STEP [19] showed homology of less than 50% to RKPTP. This result suggests that RKPTP is only distantly related to the other known cytosolic PTPases.

In conclusion, we isolated a cDNA encoding a new member of the PTPases from rat kidney. Further study is required to establish the physiological and pathophysiological significance of RKPTP in various cellular processes such as growth regulation and differentiation.

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