FEBS Letters 356 (1994) 351–356

FEBS 14918

Identification of a novel protein tyrosine phosphatase with sequence homology to the cytoskeletal proteins of the band 4.1 family

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Received 25 July 1994; revised version received 31 October 1994

Abstract Use of the polymerase chain reaction (PCR) in conjunction with Southern hybridization, using probes corresponding to known phosphatase sequences, resulted in the identification of rat cDNA clones encoding a novel protein tyrosine phosphatase which was termed rPTP2E. The cDNAs comprise 5,543 bp and predict a polypeptide of 1175 amino acids possessing a single catalytic domain at its C-terminus. The N-terminal region of the deduced polypeptide displays high sequence homology to the cytoskeleton-associated proteins of the band 4.1 family. A variant form, termed rPTP2E1, was also identified which contains the catalytic domain only. rPTP2E and rPTP2E1 were expressed in various rat tissues, particularly abundantly in adrenal glands. The catalytic domain of PTP2E was expressed in *Escherichia coli* and was shown to possess specific protein tyrosine phosphatase activity. The identification of rPTP2E suggests the existence of a subfamily of band 4.1 domain-containing PTPs which may play an important role in signalling pathway and control of cytoskeletal integrity.

Key words: Protein-tyrosine phosphatase; rPTP2E; Band 4.1 domain containing PTP; Cytoskeletal integrity; Signal transduction

1. Introduction

Protein-tyrosine phosphatase (PTP) is a rapidly expanding family of enzymes which can be divided into two classes: (i) transmembrane, receptor-like and (ii) intracellular, cytosolic PTPs. Recent advances in the identification of novel PTPs indicate that these enzymes do not act as mere negative switches of cellular processes or as 'house-keeping' enzymes antagonizing the action of PTKs; rather, they actively participate in nearly every aspect of signal transduction. Since all PTPs identified contain a conserved PTP domain it is expected that the characteristics of sequences other than the catalytic domain of these enzymes probably play a major role in determining their specific functions within the cells. Accordingly, the intracellular enzymes can be further divided into several subfamilies based on the nature of their distinctive non-catalytic regions. One such subfamily of intracellular PTPs is made up of enzymes possessing two src homology 2 (SH2) domains. Three members of the SH2 domain-containing PTPs have been cloned. They are PTP1C (also known as SH-PTP1, HCP, or SHP) [1–3], PTP2C (also designated as SH-PTP2, Syp, or PTP1D) [4–7], and *Drosophila corkscrew* (csw) [8]. As with other SH2 domain-containing proteins, the SH2 domains of these PTPs bind to the autophosphorylation sites of tyrosine kinase receptors and other tyrosine-phosphorylated proteins, thus participating in signal transduction pathways [6,7,9,10]. Another emerging subfamily of intracellular PTPs is composed of proteins possessing a large non-catalytic segment with homology to the N-terminal portion of the cytoskeleton-associated proteins of the band 4.1 family, which includes ezrin [11], radixin [12], and moesin [13]. This subfamily of PTPs is expanding and currently includes PTPH1 [14], PTP-MEG1 [15], and hPTP1E, also known as PTP-BAS and PTPL1 [16–18]. In this report, we describe the identification from rat tissues of a fourth member of this band 4.1 domain-containing PTP subfamily, a cytosolic PTP which we designated as rPTP2E.

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Abbreviations: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SH2, src homology 2; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT-PCR, reverse-transcription PCR; IPTG, isopropyl-β-D-thiogalactopyranoside; RCM-lysozyme, reduced carboxamidomethylated and maleylated lysozyme; PBS, phosphate buffered saline; bp, base pair; kb, kilobase; kDa, kilodalton; LAR, leukocyte common antigen-related protein.

2. Materials and methods

2.1. Isolation of rPTP2E cDNA

In order to obtain novel PTP cDNA sequences, the following strategy was employed. RNA prepared from mouse embryos (4 days) was subjected to RT-PCR using a pair of degenerate oligonucleotides, derived from two well-conserved regions of known PTPs. The sequence of the first oligonucleotide corresponded to the amino acid sequence DFWRMV/IWE and the second to the amino acid sequence WPDF/HGVP. The resulting RT-PCR fragments were cloned into the pGEM-T vector (Promega) to facilitate further manipulation. In order to eliminate known PTP sequences, the RT-PCR fragments were re-amplified from individual transformed bacterial colonies, separated on an agarose gel, and transferred to a nylon membrane by Southern blotting. The membrane was hybridized with a mixture of DNA probes consisting of the catalytic domains of LAR(D1), LAR(D2), HPTPα, HPTPδ, HPTPε, PTP1C and several other known PTPs [1,19,20]. The blots were then subjected to high stringency washes. The PCR fragments which exhibited a weak or no signal were analyzed by DNA sequencing. Those fragments which contained novel PTP sequences were used as probes to screen the corresponding overlapping clones in a rat cDNA library (Stratagene) made from decidual tissue, or in a mouse embryo cDNA library. DNA sequencing was determined on both strands by the dideoxy chain-termination method (T7 Sequencing Kit; Pharmacia Inc.). The anchor PCR was performed as described [16].

2.2. RNA isolation and Northern blot analysis

Total RNA or poly(A)$^+$ RNA was purified from rat tissues using the standard guanidine isothiocyanate/acid-phenol method. Approxi-

SSDI 0014-5793(94)01305-5

mately 20 μg total RNA (or 6 μg of poly(A)$^+$ RNA) from each tissue was separated on a 1% (w/v) agarose-formaldehyde gel by electrophoresis and blotted onto a nylon filter. The membrane was subsequently hybridized with ^{32}P-labelled probes corresponding to the catalytic domain of rPTP2E cDNA fragment. Hybridized mRNA was detected by autoradiography.

2.3. Expression in Escherichia coli *and phosphatase assay*

A segment encoding the entire catalytic domain of rPTP2E (amino acids 834–1175) was amplified by PCR and cloned in-frame into the bacterial expression vector pET-3c [21]. The resultant plasmid, designated pΔPTP2E, was used to transform *Escherichia coli* BL21 (DE3) cells. Expression of recombinant enzyme was carried out as described [1] except that cells were cultured at 22°C and induced by 50 μM IPTG for 24 h. The recombinant protein consists of 351 amino acids in which the first 9 residues were derived from the vector.

For the phosphatase assay, the substrate, RCM-lysozyme (Life Technologies Inc.), was tyrosine-phosphorylated with [γ-^{32}P]ATP using p60$^{c\text{-}src}$ (Oncogene Science) following the manufacturer's instructions. The induced bacteria were centrifuged, resuspended in 25 mM Tris-HCl, pH 8.0, and 5 mM DTT, and lysed by sonication. Activity assay was performed by incubating crude bacterial lysates in 25 mM, pH 8.0, 5 mM DTT, 15 mM NaCl, 10 μg/ml BSA, 8 mM $MgCl_2$ and 0.77 μM ^{32}P-labelled RCM-lysozyme at 30°C for 30 min. The reaction was stopped by addition of 400 μl of charcoal mixture (0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH_2PO_4, 4% w/v Norit A). After vortexing and centrifugation, the released radioactive P_i present in the supernatant was determined by scintillation counting. Protein determination was performed by a standard Bradford assay.

3. Results

3.1. Isolation and characterization of rPTP2E cDNA

In order to identify novel PTP sequences in mRNA preparations, RT-PCR was performed using a pair of degenerate oligonucleotide primers designed from the conserved regions of the catalytic domains of known tyrosine phosphatases. The amplified PCR fragments, about 250 bp in length, were cloned into the pGEM-T vector (Promega). The putative PTP sequences were individually re-amplified from each clone with T7 and Sp6 primers flanking the inserts, and analyzed by Southern blotting. The membranes were hybridized at high stringency with a mixture of probes derived from the catalytic domains of previously identified PTP cDNAs. Clones displaying weak or no hybridization signal were analyzed by DNA sequencing. Out of approximately one thousand primary clones, a few appeared to encode novel PTP sequences. One such identified PCR fragment was used to screen a rat cDNA library.

Several overlapping clones were obtained which constituted a cDNA sequence of 5,543 nucleotides. The cDNA sequence contains a single open reading frame starting from nucleotide 266, predicting a protein of 1175 amino acid residues which we termed rPTP2E (Fig. 1A). The absence of an N-terminal signal peptide and putative transmembrane sequence suggests that rPTP2E is a cytosolic PTP. The phosphatase domain, consisting of approximately 240 amino acid residues, is located at the extreme carboxyl end of the protein. This domain shares about 40% amino acid sequence identity with the catalytic domains of several known PTPs. The large middle portion of rPTP2E, encompassing amino acid residues 349–916, does not contain significant homology to known protein sequences. At the N-terminus, a segment of about 350 amino acids displays 25–30% identity to the N-terminal portion of the cytoskeletal protein family, including moesin [13], ezrin [11], radixin [12], merlin [22,23] and the protein band 4.1 [24]. This region also shares sequence homology with three human tyrosine phosphatases, PTPMEG1 [15], PTPH1 [14] and the recently cloned hPTP1E/PTP-BAS/PTPL1 [16–18] with 34, 30, and 25% identity, respectively.

In addition to the clones encoding the 1175 amino acid polypeptide of rPTP2E, a related cDNA clone was also identified from the cDNA library. This cDNA was 3064 nucleotides in length and differed from the longer cDNA (rPTP2E) in the first 299 base pairs which were unique to the shorter sequence (Fig. 1B and Fig. 2). Starting at position 300, the sequence of this cDNA clone was identical to that of the longer cDNA clone rPTP2E (position 2779 to the end). The first in frame ATG initiation codon within this shorter cDNA clone was located at nucleotides 304–306 which corresponds to the methionine residue 840 of the rPTP2E protein (nucleotides 2783–2785 of the 5.6 kb cDNA). The predicted protein from this 3.1 kb cDNA consists of the last 335 amino acid residues of the rPTP2E protein, which constitute almost exclusively the catalytic domain. This variant polypeptide was termed rPTP2E1. To verify the presence of the variant rPTP2E1 transcripts in rat tissues, PCR and anchor PCR were performed using reverse-transcribed rat total RNA with primers specific to rPTP2E1 (Fig. 2). Sequencing of both the PCR and anchor PCR products confirmed that rPTP2E1 transcripts were indeed present in rat RNA.

Several mouse cDNA clones homologous to PTP2E were also identified from an embryonic mouse cDNA library. Partial sequence determination of these clones revealed an amino acid sequence identity of over 96% between the two species (data not shown).

3.2. Expression of rPTP2E in rat tissues

The expression of rPTP2E in rat tissues was assessed by Northern blot analysis (Fig. 3). Total RNA prepared from various rat tissues was hybridized to a cDNA probe corresponding to the catalytic domain. Two major species of mRNA, 3.1 kb and 5.6 kb, were readily detected in most tissues with the exception of the spleen. The sizes of these two major species of mRNA were consistent with that of rPTP2E and rPTP2E1 cDNA clones described above. The distributions of

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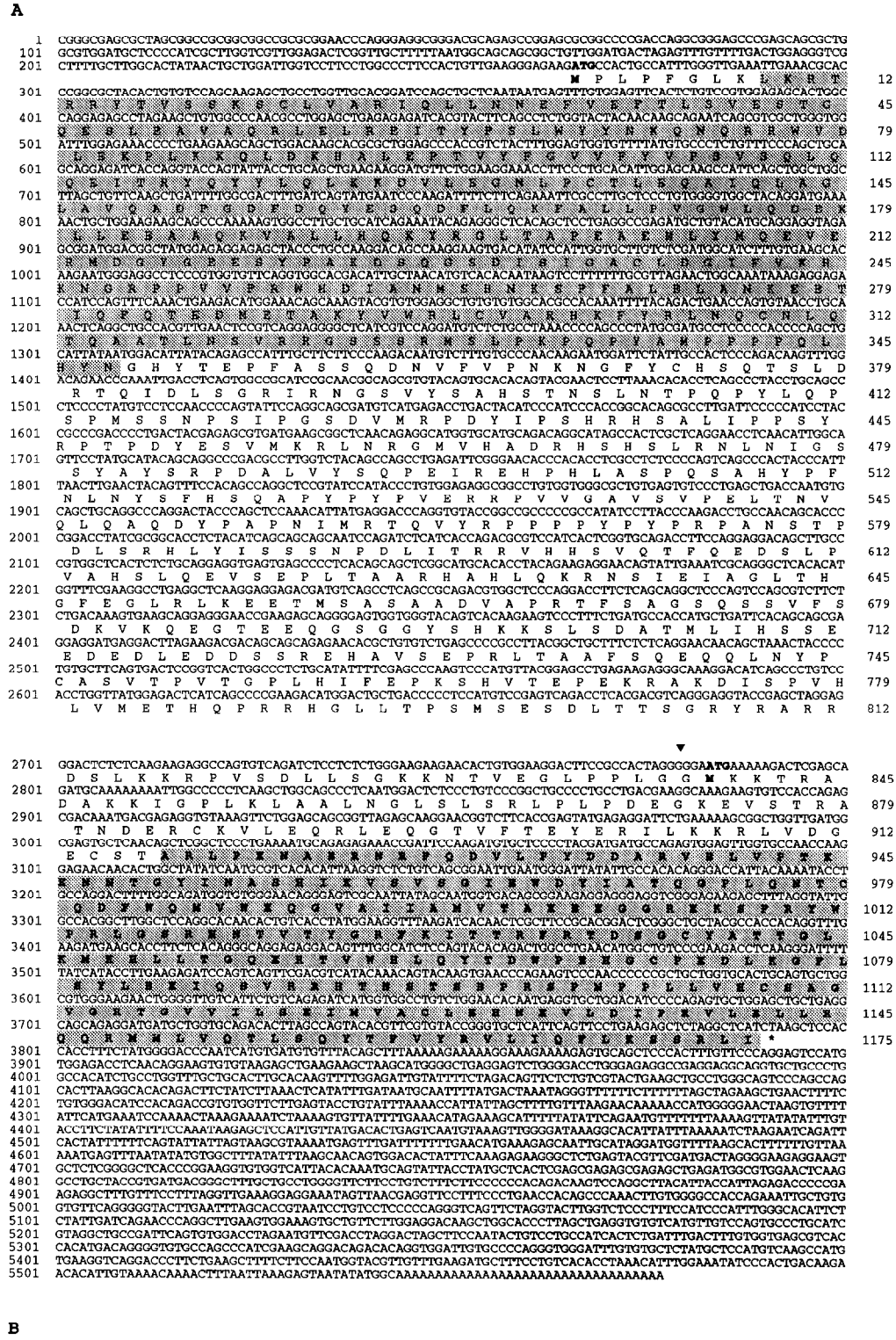
Fig. 1. Nucleotide and deduced amino acid sequence of the rPTP2E and rPTP2E1 cDNAs. (A) rPTP2E. The nucleotides are numbered from the 5′ end of the 5.6 kb cDNA clone. The first ATG codon in the open reading frame at position 266–268 and another ATG codon at position 2783–2785 which is used as the initiation codon of its variant rPTP2E1 (see Fig. 1B), are shown in bold letters. The arrowhead indicates the position from which the nucleotide sequence is identical to that of its variant rPTP2E1 (see Fig. 1B). The band 4.1 domain is marked by the shaded area (amino acid residues 9–348). The catalytic domain is presented in bold letters on a shaded background (amino acid residues 917–1175). (B) The 5′ non-coding nucleotide sequence of rPTP2E1. The nucleotides are numbered from the 5′ end of the 3.1 kb cDNA clone. The first ATG codon in the open reading frame at position 304–306 is marked in bold letters. The number (840) in parentheses refers to amino acid residue Met-840 of rPTP2E protein in A. The dashed lines following both the nucleotide and amino acid sequences represent their identity to the rPTP2E cDNA clone (A). The arrowhead indicates the position from which the nucleotide sequence is identical to that of rPTP2E (starting from position 2779 in A).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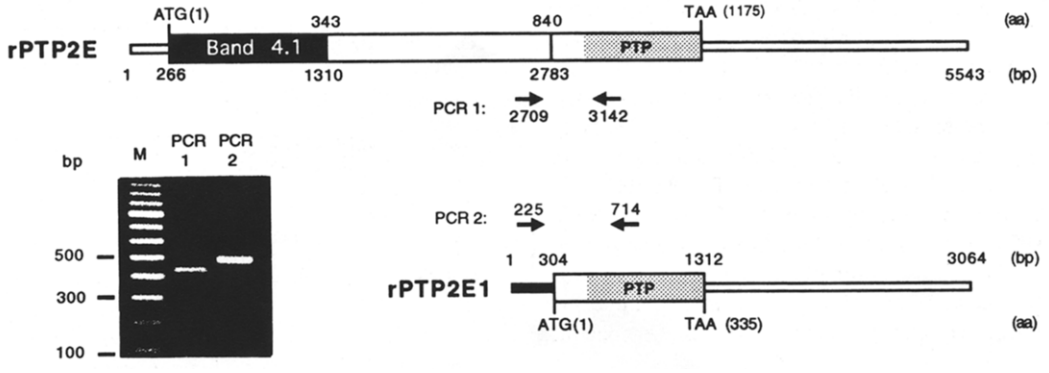


Fig. 2. Schematic comparison of the structures of rPTP2E and rPTP2E1. The boxes represent the coding regions; the thinner boxes represent 5′ and 3′ untranslated regions. The catalytic domain, the band 4.1 domain, and the numbering of the nucleotides and amino acid residues in each clone are indicated. Arrows indicate the positions of the primers designed for RT-PCR of rPTP2E and rPTP2E1 segments, respectively. Inset: RT-PCR results. Lane 1, from rPTP2E (PCR1); Lane 2, from rPTP2E1 (PCR2).

the species, and the expression level of mRNA was variable from one tissue to another. Both species of mRNA were present in heart, muscle and adrenal gland with the strongest signal for both species in adrenal gland. Only the short form (3.1 kb) was expressed in the ovary and testis, whereas brain, uterus and lung expressed only the long form (5.6 kb). In the liver, in addition to the long form (5.6 kb species), a 2.4 kb mRNA also strongly hybridized to the probe. However, the corresponding cDNA clones to the 2.4 kb mRNA species have not been identified yet.

3.3. Expression of the recombinant enzyme

To investigate whether the cDNA of PTP2E encodes a functional enzyme, the C-terminal portion of PTP2E, which contains the entire catalytic domain of the phosphatase, was cloned into the bacterial expression vector pET-3c. The recombinant protein expressed in induced *E. coli* cells, although accounting for about 15% of total bacterial cellular proteins (Fig. 4 inset, lane +), was found to be nearly totally insoluble. The 39 kDa band of the recombinant protein observed in induced cells could not be detected in the supernatants by Coomassie blue-stained SDS-PAGE (data not shown). However, the supernatants prepared from the induced cells harbouring pΔPTP2E plasmid displayed a more than 10-fold increase in tyrosine phosphatase activity in comparison with that resulting from cells transformed by the vector alone (Fig. 4) when labelled RCM-lysozyme was used as substrate. The activity of the recombinant enzyme toward this substrate, although relatively low, was nearly completely inhibited by 1 mM sodium vanadate, a characteristic of protein tyrosine phosphatases (data not shown). The recombinant protein did not show an apparent activity toward *p*-nitrophenyl phosphate, an artificial, non-natural substrate for protein tyrosine phosphatases. These results indicate that PTP2E may specifically interact with its physiological substrate(s).

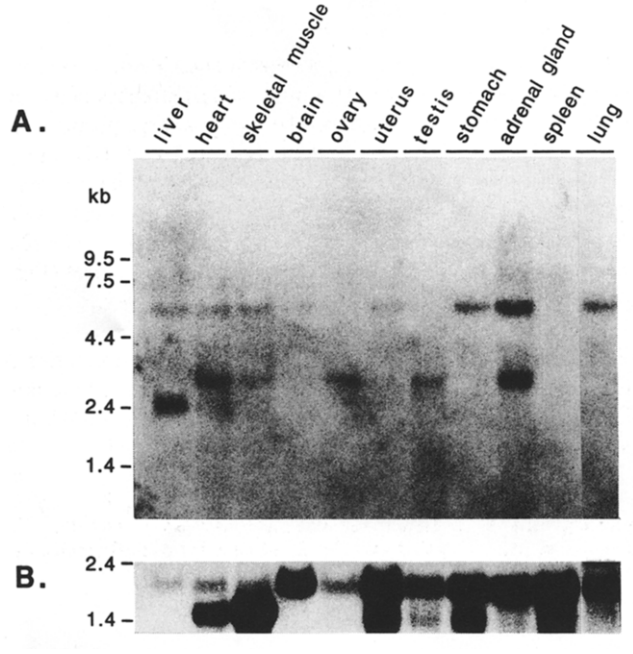


Fig. 3. Analysis of rPTP2E mRNA abundance by Northern blotting. RNAs were prepared from various rat tissues. Each lane contains 20 μg of total RNA. The expressed mRNA level of rPTP2E in the tissues was examined by hybridization with a 275 bp fragment probe prepared from the catalytic domain (nucleotides 3206–3475) of rPTP2E (A). A β-actin probe was used as a control to check the integrity of the mRNA (B). The numbers on the left refer to the size of RNA markers given in kb.

4. Discussion

To identify novel PTP sequences we have used known PTP probes hybridized to cloned RT-PCR fragments on Southern blots. Out of several hundred primary candidates, one PCR fragment did not hybridize to the probes used. Nucleotide sequence analysis confirmed that this PCR fragment corresponded to a new PTP sequence. Screening a rat cDNA library with this PCR fragment as a probe resulted in the identification of several overlapping clones encoding a new soluble phosphatase which we termed rPTP2E. A variant of rPTP2E, termed rPTP2E1, was also identified. rPTP2E1 is a truncated version of rPTP1E containing almost exclusively the phosphatase catalytic domain of rPTP2E. This variant appears to be produced by the transcription of the same gene, 5′ but from a different promoter, and a new site of initiation, since the 5′ non-coding region of the cDNA is completely different from that encoding the larger form of the protein. Northern blot analysis revealed the presence of three rPTP2E RNA transcripts in rat tissues. The cDNA clones of rPTP2E and rPTP2E1 correspond to the

species of 5.6 kb and 3.1 kb mRNA, respectively. cDNA clones corresponding to the 2.4 kb transcript observed in the liver were not identified in the cDNA libraries screened.

The N-terminal portion of rPTP2E consisting of approximately 350 amino acid residues, displays homology to the band 4.1 protein family which includes ezrin [11], radixin [12], moesin [13], talin [25], merlin [22–23] and three PTPs, PTPH1 [14], PTPMEG1 [15] and the recently cloned hPTP1E/PTP-BAS/PTPL1 [16–18]. The importance of this protein family has been emphasized recently with the finding that the tumor suppressor neurofibromatosis type 2 gene (NF2) encodes a cytoplasmic protein with striking sequence similarity to several members of this protein family [22,23].

The subcellular localization of some of these proteins has been studied. It has been shown that several members of the band 4.1 domain-containing proteins co-localize with the cortical actin cytoskeleton where they interact with both the cell membrane and the actin cytoskeleton [26]. The presence of a highly homologous domain in rPTP2E suggests that this phosphatase, as well as the other members of this subfamily of tyrosine phosphatases, are most likely targeted to the interface between the plasma membrane and the cytoskeleton, where they may regulate cellular signal transduction pathways through dephosphorylation of other proteins present within this subcellular compartment.

During the review of this paper, the sequence of a related protein, PTPD1, was reported by Møller et al. [27]. Amino acid sequence comparison revealed that rPTP2E and human PTPD1 share a great deal of sequence identity, particularly within their N-terminal band 4.1 domains and their C-terminal catalytic

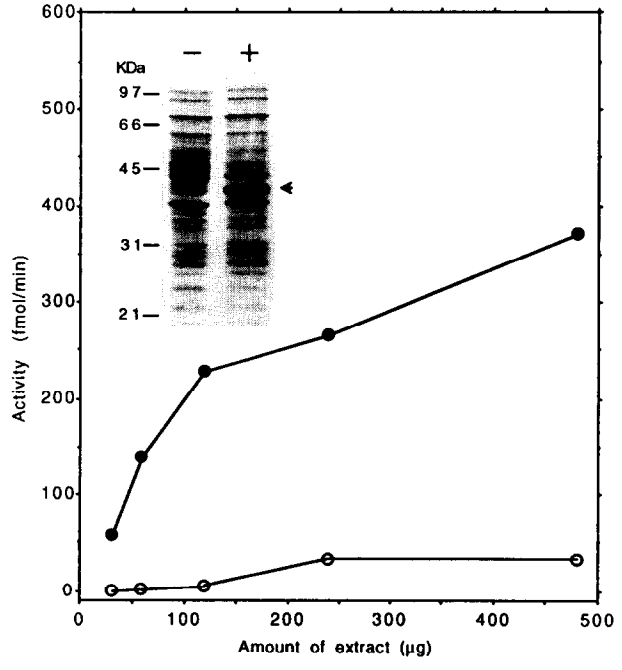
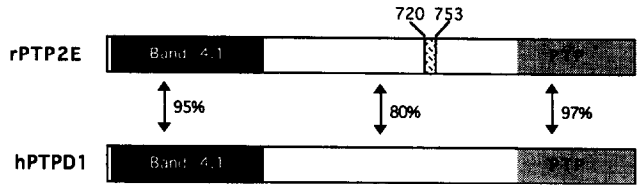


Fig. 4. Phosphatase activity of recombinant PTP2E expressed in bacteria. Phosphatase activity, expressed as increased release of P_i in fmol /min from ^{32}P-labelled RCM-lysozyme, is presented as a function of the amount of bacterial extracts. ●, cells transformed with pΔPTP2E; ○, cells transformed with pET-3c vector alone. The inset shows a Coomassie blue-stained gel (SDS-PAGE) of *E. coli* cells transformed with pΔPTP2E plasmid. Lane +, IPTG induced cells; lane −, non-induced cells. The 39 kDa band corresponding to the recombinant enzyme is indicated by the arrow.



A

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rPTP2E   351 YTEPFASSQDNVFVPNKNGFYCHSQTSLDRTQIDLSGRIRNGSVYSAHSTNSLNTPQPVL
hPTPD1   351     Y       L     Q  Y             A    FN                N

rPTP2E   411 QPSPMSSNPSIPGSDVMRPDYIPSHRHSALIPPSYRPTPDYESVMKRLNRGMVHADRHSH
hPTPD1   411              T             L          V                T   Q     L    E Q

rPTP2E   471 SLRNLNIGSSYAYSRPDALVYSQPEIREHPHLASPQSAHYPFNLNYSFHSQAPYPYPVER
hPTPD1   471                       A                AQ P  AA  C  S S       PS      A

rPTP2E   531 RPVVGAVSVPELTNVQLQAQDYPAPNIMRTQVYRPPPPYPYPRPANSTPDLSRHLYISSS
hPTPD1   531                    A          S                    P

rPTP2E   591 NPDLITRRVHHSVQTFQEDSLPVAHSLQEVSEPLTAARHAHLQKRNSIEIAGLTHGFEGL
hPTPD1   591                                                    Q H       V   S  L

rPTP2E   651 RLKEETMSASAADVAPRTFSAGSQSSVFSDKVKQEGTEEQQSGGYSHKKSLSDATMLIHS
hPTPD1   651      R L       E     AV V     P     TERTQR   P   AEGLR G

rPTP2E   711 SEEDEDLEDDSSREHAVSEPRLTAAFSQEQQLNYPCASVTPVTGPLHIFEPKSHVTEPEK
hPTPD1   711      E   -  FEEESG  RAP  ARAREPRPGLAQDP  GCPRVLLA       L    A   PDA

rPTP2E   761 RAKDISPVHLVMETHQPRRHGLLTPSMSESDLTTSGRYRARRDSLKKRPVSDLLSGKKNT
hPTPD1   760  MM S     RTTA AQR W D     M                                                I

rPTP2E   821 VEGLPPLGGMKKTRADAKKIGPLKLAALNGLSLSRLPLPDEGKEVSTRATNDERCKVLEQ
hPTPD1   820                  V                              V           A           I

rPTP2E   881 RLEQGTVFTEYERILKKRLVDGECST
hPTPD1   880         M
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B

Fig. 5. (A) Schematic comparison of the sequence homology of rPTP2E and hPTPD1. The band 4.1 domains, the catalytic domains and the middle portions are represented and the percentage of identity between the segments of the two proteins is indicated. Numbers refer to the positions of the amino acid residues of rPTP2E. (B) Detailed sequence comparison of amino acid residues 351–906 of rPTP2E with the homologous segment of hPTPD1. The amino acid sequences of the middle portion of rPTP2E and hPTPD1 are aligned. Only the residues of hPTPD1 differing from those of rPTP2E are presented. The minus symbol indicates a missing residue in hPTPD1.

domains which share 95% and 97% sequence identity, respectively (Fig. 5A). There are 141 amino acid differences between the sequences of the two proteins. However, a large proportion of these differences is located in the middle region of the protein where the two polypeptides have an overall 80% sequence identity. Interestingly, there is a segment in the middle portion of the two proteins (amino acid residues 720–753 of rPTP2E) where the two proteins display entirely different sequences (Fig. 5B). At present it is unknown whether the sequence difference in this region of the two proteins is the result of alternative splicing of exons within the rat and human genes. Nevertheless, the sequence differences observed between rPTP2E and human PTPD1 may imply the existence of yet further variation within the subfamily of the band 4.1 domain-containing PTPs.

Acknowledgements: We thank N. Jolicoeur for art work, X. Feng, P. Bouchard and F. Bélanger for technical assistance, and Dr. C. Richardson, Mr. J. Vialard and Dr. R. Mosser for critical comments on the manuscript.

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