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INTRODUCTION

Protein phosphorylation, the transfer of phosphoryl groups from ATP on intracellular proteins, is recognized as a key event in the transduction of extracellular signals [1, 2]. Subsequent removal of phosphoryl groups from target proteins by protein phosphatases has long been considered a housekeeping process without any regulatory role in cell signalling. However, for many different organisms, such as Yersinia [3], yeast [4], Dictyostelium [5, 6], Drosophila [7] and mouse [8, 9], recent experiments have now revealed important roles for protein tyrosine phosphatases (PTPases) in the regulation of development and cellular proliferation. As a consequence, it has now become clear that the interplay of kinases and phosphatases is crucial for actuating cellular responses to extracellular signals. Structural information on PTPases has accumulated rapidly over the last few years, and many have been found to be transmembrane receptor-type molecules [10–12]. These enzymes thus represent a novel repertoire of signalling proteins the precise role of which in cell physiology, growth control and pathogenesis still has to be determined.

To obtain the molecular tools for further study of the bioactivity of PTPases in mouse development, we have cloned several PTPase sequences from murine brain employing degenerate oligonucleotide primers and PCR techniques. A novel PTPase (PTP-SL) was found and its full-length cDNA was isolated and characterized.

MATERIALS AND METHODS

PCR and subcloning of PTPase domains

Degenerate oligonucleotide primers 1 and 2 were based on consensus sequences for highly conserved amino acid stretches within the catalytic domains in PTPases: primer 1, 5'-GA(C/T)TTT(C/T)TGGG(A/C)(A/G/T)(A/G)ATG(A/G/T)(A/C/T)TGGG(G/C)A-3'; primer 2, 5'-C(G/T)CCC(A/T)(A/G)-C(A/G/C/T)CC(A/T)G(G/A/C/T)CT(A/G/CAGTTG-3'. DNA of a mouse brain cDNA phage library (Stratagene) was used as a template for PCR. Primers 1 and 2 (final concentration 7 ng/µl) were added to a 100 µl reaction mixture containing 20 mM Tris/HCL (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.01 % BSA, all four dNTPs (each at 250 μM), 2 units of Taq polymerase (Perkin-Elmer Cetus) and 10 ng of phage DNA. Thirty-five cycles were performed on a Perkin-Elmer thermal cycler; each cycle involved incubation at 94 °C for 0.5 min, at 37 °C for 0.5 min and at 72 °C for 1 min. The PCR products were treated with proteinase K as described previously [13]. After DNA end-repair using Klenow (large fragment) DNA polymerase, the fragments were 5'-phosphorylated with T4 polynucleotide kinase and ATP, and analysed on a 1.5 % low-gelling-temperature agarose gel. Fragments of 350–400 bp were excised and subcloned into the Smal site of plasmid pBlueScript by standard protocols [14]. Sequences were determined using the double-stranded DNA dideoxy sequencing method [15].

Isolation and sequencing of mPTP13 cDNAs

The mPTP13 PCR fragment was isolated, labelled radioactively by random priming [16], and used to screen a mouse brain λ-ZAPII cDNA phage library (Stratagene). Hybridization conditions were those of Church and Gilbert [17]: membranes were preincubated in hybridization buffer [7 % SDS, 0.5 M sodium phosphate buffer (pH 7.4), 1 mM EDTA] for 10–60 min at 65 °C. After probe denaturation and addition, membranes were hybridized overnight at 65 °C. Washing at high stringency [0.1 % SDS, 0.04 M sodium phosphate buffer (pH 7.4), 1 mM EDTA] was performed three times at 65 °C for 20 min. Autoradiography was on Kodak X-Omat S1 films at —70 °C for 1–2 days using Dupont Cronex intensifying screens. Positive phages were plaque-purified and inserts were rescued as pBlueScript SK plasmids according to the manufacturer's protocols. Nucleotide sequences were determined using the DNAase shotgun strategy [18] in combination with the double-stranded DNA dideoxy sequencing method [15]. DNA sequence gel readings were recorded, compared, edited and assembled using the IG-SUITE 5.35 package (Intelligenetics, Mountain View, CA, U.S.A.). Deduced protein sequences were analysed using the GCG package [19] and the SIGSEQ program [20] provided by the Dutch CAOS/CAMM Center.

Abbreviations used: PTPase, protein tyrosine phosphatase; poly(A)*, polyadenylated; GST, glutathione S-transferase.

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Figure 1  Alignment of deduced amino acid sequences from PCR-derived mouse brain PTPase cDNA clones

The number above the alignment indicates the relative position within the region which starts immediately after the sequence DPWRM(V/I)V(G/D) found in most PTPase domains. The region just before the stretch that contains the essential cysteine residue [HCSAG(V/I)]GR in the catalytic domain. The gaps introduced was kept to a minimum and the gap around p62 was introduced between amino acid residues the corresponding triplets of which are located in different exons of the LCA gene [25,26]; the other two gaps were arbitrarily chosen. The position at which ten or more PTPase domains share identical residues are shown on a shaded background. mPTP1β is identical with part of PTP-1, the mouse homologue of PTP1B [27,28], m and mLRP-2 were identical with the two LRP phosphatase domains [29,30]. mPTPβ-1 represents the first phosphatase domain of mPTPβ [31], mPTPβ-1 is identical with PTP-tyR [27], the homologue of the first phosphatase domain in human HTPPβ [32], mLAR-1 and mLAR-2 very closely resemble the first and second phosphatase domain respectively in human [33] and mLAR. Likewise, mPTP-1 is the mouse homologue of human BPTP-1 [35] and rat PTP-2 [36], PTPNE-3 [37], PTPγ [38], CPTP[39] and PTP-P1 [40]. mPTPβ is the homologue of H [32], and mPTPβ-2 strongly resembles the second phosphatase domain in human HTPPβ [32]. Six of these cDNA fragments have been published [41]. Finally, three clones (mPTP13, mLAR and mPTP38) are different from the phosphatase domains currently published.

RNA isolation and Northern-blot analysis

Total RNA was isolated from various sources by the LiCl/urea method [21] and stored as alcohol precipitates. Selection of polyadenylated [poly(A)]tRNA on oligo(dT)-cellulose was as described previously [14]. Poly(A)+ RNA (2 μg) was resolved on a 2.2 M formaldehyde/1 % (w/v) agarose gel, transferred to Hybond-N+ membranes (Amersham) [14] and fixed by u.v. irradiation (Stratagene type; Stratagene). PCR-derived PTPase inserts, labelled by random priming [16], were used as probes. Hybridization conditions were as described above. Autoradiography was on Kodak X-Omat S1 films for 1–4 days at −70 °C using Du Pont Cronex intensifying screens.

Assay of phosphatase activity

A 1.9 kb SmaI–XhoI fragment (starting at nt. 866) of clone mPTPβ-3 was treated with Klenow large-fragment DNA polymerase to create a blunt-ended fragment encoding the last amino acids of PTP-SL. This fragment was cloned into the S site of pGEX-2T [22] to create pGEX-SL. Escherichia coli D (Gibco-BRL) transformed with pGEX-SL or the empty vector pGEX-2T were grown in 10 ml of Luria broth containing 100 μg/ml ampicillin until A550 reached 0.7, and were induced with 0.1 mM isopropyl thiogalactoside for 4 h at 37 °C. Bacteria were transferred to Eppendorf tubes, pelleted, washed once with 200 μl of cold buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA), and resuspended in 250 μl of buffer (150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, pH 7.3) containing Triton X-100 and 0.4 mM phenylmethylsulphonyl fluoride. After the addition of 25 μl of lysozyme solution (10 mg/ml), mixture was incubated for 15 min at 37 °C. After three freeze-thaw cycles, 2 μl of DNAse I (1 μg/μl in 1 M MgCl2) was added to the lysate and the mixture incubated for 15 min at 37 °C. After centrifugation (10 min at 10000 g) the supernatant was transferred to a new tube and protein concentration de
deduced protein sequences for these three clones clearly display corresponding to an mRNA size of around 8 kb are found in mPTPiv-2). The consensus features of tyrosine phosphatase domains (Figure RNA isolated from 13.5-day embryos and brain, muscle, heart domains were found: mPTP13, mPTP14 and mPTP38. Still, the In contrast, mPTP14 is more regions that were identical with reported murine PTPases [27-31].

In vitro translation

The mPTP13-6 cDNA clone was linearized with XhoI and used for in vitro transcription with T3 RNA polymerase (Pharmacia) according to the manufacturer’s protocols. RNA (200 ng) was heated for 10 min at 67 °C and added to a rabbit reticulocyte lysate (Promega) for in vitro translation in the presence or absence of canine pancreatic microsomal membranes (Promega) as described by the manufacturer. The [35S]methionine-labelled products were analysed by SDS/PAGE on a 10 %, polyacrylamide gel [24] and visualized, after treatment with dimethyl sulphoxide/2,5-diphenyloxazole and drying of the gel, by autoradiography on Kodak X-Omat S1 film for 16 h.

RESULTS

Cloning of three novel PTPase cDNA fragments

To isolate murine cDNA clones encoding PTPases, we designed denerate oligonucleotide primers based on sequences conserved in the core region of the catalytic phosphatase domain of most known PTPases. Using mouse brain cDNA as template, 29 clones with PTPase domain-like inserts, grouped into 13 different sequences, were identified (Figure 1). Computer-assisted comparison with published PTPase domain sequences was performed to reveal the possible identity of the clones. Five sequences (mPTP1B, mLRP-1 and -2, mPTPβ-1 and mPTPα-1) contained regions that were identical with reported murine PTPases [27-31]. Five sequences (mLR-1, mLR-2, mBPTP-1, mPTPβ and mPTPα-2) probably represent mouse homologues of human or rat PTPases [32-39] for which no mouse clones have been identified until now. Finally and most interestingly, three clones with only weak homology to currently published phosphatase domains were found: mPTP13, mPTP14 and mPTP38. Still, the deduced protein sequences for these three clones clearly display the consensus features of tyrosine phosphatase domains (Figure 1). We conclude therefore that these sequences represent new PTPase family members.

To investigate the nature and expression patterns of the transcripts from which these three novel PTPase cDNA fragments originate, PCR fragments were used as probes on Northern blots containing RNA samples of various murine tissues. The mPTP13 probe hybridized to mRNA species of 4.1 and 3.2 kb in adult brain. A very weak signal of the 4.1 kb mRNA was detectable in 13.5-day embryos (Figure 2a). Also mPTP38 is exclusively expressed in brain, and originates from a single 4 kb mRNA species (Figure 2c). No hybridizing signal could be obtained with RNA isolated from 13.5-day embryos. Southern blots from different digests of murine genomic DNA yield distinct hybrid-
nature of this novel PTPase. While this manuscript was in preparation, Maekawa and co-workers [45] published a human sequence with close homology to mPTP14. They predict an open reading frame which contains a band-4.1-like domain and, in addition, the longest insert (2.9 kbp) present in clone PTP13-6 was sequenced completely. All other clones were shown by end-in sequencing to represent shorter versions of the same transcript.

Analysis of mPTP13 cDNA

Northern-blot analysis had suggested the possibility of developmentally regulated alternative splicing for the mPTP13 coding region. Therefore we conclude that mPTP13-6 represents a full-length transcript as a probe. Eleven overlapping clones were obtained spanning 549 residues and is preceded by several in-frame stop codons. Therefore we conclude that mPTP13-6 represents a full-length transcript as a probe. Eleven overlapping clones were obtained spanning 549 residues and is preceded by several in-frame stop codons.

Figure 3 Complete nucleotide sequence of mPTP13-6 and deduced amino acid sequence of PTP-SL

The asterisk indicates a stop codon. The four putative translation initiation codons discussed in the text and the preceding in-frame stop codons are underlined. The signal sequence (Sig) transmembrane domain (TM) and poly(A)+ signal (pA) are indicated by open boxes. The arrow marks the putative cleavage site within the signal sequence. The sequence present in mPTP13 is shown on a shaded background.

Responding gene. To obtain full-length cDNA clones, a mouse brain cDNA library was screened with the PCR-derived mPTP13 fragment as a probe. Eleven overlapping clones were obtained and the longest insert (2.9 kbp) present in clone PTP13-6, was sequenced completely. All other clones were shown by end-in sequencing to represent shorter versions of the same transcript species. The nucleotide sequence and deduced amino acid sequence are shown in Figure 3. An open reading frame is found encoded between the recombination sites.
Table 1 Phosphotyrosine phosphatase activity of GST–SL fusion protein in the presence or absence of various potential inhibitors

<table>
<thead>
<tr>
<th>Protein</th>
<th>Modifier</th>
<th>Activity (% of GST–SL)</th>
</tr>
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<tbody>
<tr>
<td>GST</td>
<td>—</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>GST–SL</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>GST–SL</td>
<td>0.5 mM vanadate</td>
<td>40</td>
</tr>
<tr>
<td>GST–SL</td>
<td>2.5 mM vanadate</td>
<td>5</td>
</tr>
<tr>
<td>GST–SL</td>
<td>0.1 mM phenylarsine oxide</td>
<td>42</td>
</tr>
<tr>
<td>GST–SL</td>
<td>0.1 mM ZnCl₂</td>
<td>96</td>
</tr>
<tr>
<td>GST–SL</td>
<td>1.0 mM ZnCl₂</td>
<td>98</td>
</tr>
<tr>
<td>GST–SL</td>
<td>0.1 /μM okadaic acid</td>
<td>97</td>
</tr>
<tr>
<td>GST–SL</td>
<td>1.0 /μM okadaic acid</td>
<td>102</td>
</tr>
</tbody>
</table>

Computer analysis of the deduced PTP–SL protein sequence revealed a signal sequence and a hydrophobic transmembrane region, indicating that PTP–SL is a type-I transmembrane protein (Figure 3). To test whether the predicted signal sequence cleavage site [20] is used, an in vitro translation experiment was performed in the presence of pancreatic microsomes. Although the control protein (β-lactamase) was correctly processed, no change in mobility could be observed for PTP–SL (results not shown). Also the use of a truncated version of PTP–SL, made by introducing a stop codon after residue 260, did not show altered mobility as a result of signal-peptide cleavage. We can therefore conclude that either the PTP–SL signal sequence is not normally cleaved or the specific endopeptidase involved is not present or active in the microsomal fraction used. Future experiments employing peptide-specific antibodies will be necessary to reveal the precise processing and membrane orientation of PTP–SL.

**DISCUSSION**

Using degenerate oligodeoxynucleotides and PCR, probes were obtained for three novel putative PTPases. For one of these, mPTP13, Northern-blot analysis suggested developmental regulation of the corresponding brain-specific primary transcript. A full-length cDNA was subsequently isolated from a mouse brain cDNA library, representing the smaller, 3.2 kb, messenger and encoding PTP–SL. In embryonic tissue only the larger, 4.1 kb, mRNA species could be detected with the mPTP13 probe (Figure 2a). The length and terminal sequences of the inserts from the different cDNA clones indicated that all originate from the shorter transcript. Using several different cDNA fragments as probes on Northern blots of murine brain mRNA, we attempted to reveal internal sequence differences between the two transcripts. All probes, however, gave identical hybridization patterns (results not shown) making it very difficult to speculate on the origin of the two hybridizing mRNA species. They might result from the use of alternative promoters, differential splicing or polyadenylation, or the expression of recently duplicated genes. Southern-blot analysis of mouse genomic DNA digests reveals a rather simple hybridization pattern (results not shown) arguing against the latter possibility. Alternative splicing is a more likely explanation because this phenomenon has been previously demonstrated for many PTPase genes. Interestingly, in rat brain samples, both a 4.4 kb and a more prominent 3 kb mRNA species are recognized by STEP-specific probes and these have been suggested to be the result of alternative splicing of transcripts derived from a single gene [46]. Clearly, a more
detailed characterization of the PTP-SL gene structure or cloning of the larger mRNA species will be necessary to establish the precise relationships between the two transcripts.

From the mPTP13-6 cDNA sequence it can be deduced that PTP-SL is a transmembrane receptor-type PTPase containing one conserved phosphatase catalytic domain. This is a rather unusual conformation as single phosphatase domains are mainly restricted to cytoplasmic PTPases, receptor-type PTPases consisting having two tandem repeated domains. Thus far, only two exceptions have been reported: the receptor-type PTPases HPTPβ [32] and PTP-PS [40]. PTP-PS lacks the second phosphatase domain that is present in PTP-P1 because of alternative splicing of a single precursor transcript. In the light of this, it will be interesting to investigate whether the 4.1 kb transcript encodes a PTP-SL isoform with two phosphatase domains. The extracellular portions of PTP-PS, PTPβ and PTP-PS are completely different. PTP-PS contains four fibronectin type-III repeats and three immunoglobulin-like domains. HPTPβ has eight fibronectin type-III repeats. In contrast, PTP-SL has a relatively short extracellular part (Figure 3) which displays no obvious similarities to other proteins.

Many receptor-type molecules, such as the cytokine receptors, have cytoplasmic portions that do not appear to contain domains with recognizable catalytic functions. These ligand-binding molecules are thought to mediate the actual signalling effect via association, direct or indirect, with proteins that do contain such catalytic signalling functions [49]. PTP-SL has a rather short extracellular domain, like the transmembrane PTPases LRP/HPTPα and HPTPβ [32], and perhaps lacks ligand-binding capability. It is tempting to speculate that PTP-SL may be a ligand of this type. Future experiments will be aimed at identification of other transmembrane proteins contributing this ligand-binding feature. We thank Paul Mier and Joost Schalkwijk for mouse keratinocytes and Am van den Balka, M., Streuli, M. and Salto, H. (1988) J. Immunol. 141, 2781–2787.

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