Molecular cloning and characterization of PTPπ, a novel receptor-like protein-tyrosine phosphatase

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Novel cDNAs encoding a receptor-like protein-tyrosine phosphatase (rPTP) have been isolated from human breast tumour cells and foetal brain. The predicted protein of ~ 160 kDa, called PTPπ, comprises an extracellular portion with a MAM (meprin–A5 antigen–PTPμ) domain, an IgG-like domain and four fibronectin III-like repeats, a hydrophobic transmembrane domain and an intracellular portion consisting of two PTP catalytic units. The predicted amino acid sequence shows high identity with those of the two homophilic binding rPTPs, PTPμ and PTPκ. A variant of PTPπ potentially encoding a protein lacking three amino acids within the N-terminal tyrosine phosphatase domain has been identified. Reverse transcription-PCR has been used to confirm the expression of the variant in human foetal brain tissue. Expression analysis has shown that PTPπ is expressed in a variety of tissue types. Both forms of the N-terminal catalytic domain, the C-terminal catalytic domain and both catalytic domains in tandem were expressed in bacteria as fusion proteins. Intrinsic phosphatase activity was detected for all protein products with an artificial substrate. The fusion protein comprising both domains in tandem was also shown to dephosphorylate purified autophosphorylated epidermal growth factor receptor *in vitro*.

INTRODUCTION

The role of reversible protein-tyrosine phosphorylation in the control of signal transduction, cell proliferation and differentiation has been well established [1]. Many of the processes in which tyrosine phosphorylation is implicated involve the transduction of a signal via the cell membrane. Protein-tyrosine phosphatases (PTPs) regulate cellular function by dephosphorylating phosphotyrosine residues on intracellular substrates. Two subfamilies of PTPs have been identified, comprising receptor-like and intracellular forms [1]. The receptor-like PTPs (rPTPs) have in common a single hydrophobic transmembrane domain and, with the known exceptions of PTPβ and DPTP10D, possess two tandem sequences homologous to PTP catalytic domains, of which at least one shows catalytic activity [1]. The rPTPs are further subdivided into six main types on the basis of their extracellular domains [1]. The type I rPTP, CD45, has a heavily glycosylated external segment that includes a cysteine-rich region. Type II rPTPs contain two intracellular phosphatase catalytic domains, with external domains comprising fibronectin III-like repeats and/or IgG-like domains, suggesting a possible role in cell adhesion by analogy with the N-CAM superfamily of adhesion molecules [2]. The extracellular domains of type III rPTPs contain only fibronectin-like repeats and are possibly capable of mediating heterophilic interactions with ligands. Type IV rPTPs are characterized by their extremely short extracellular domains. Type V rPTPs possess an extracellular domain homologous to carbonic anhydrase [3]. A further family of rPTP-like proteins has been identified from mouse and human insulinoma and an involved (containing metastatic breast tumour) axillary lymph node (P. D. Smith, C. Laughton, K. Barker, J. Wang and M. R. Crompton, unpublished work), the members of which, while highly similar to rPTPs, appear to lack catalytic activity [4–6].

The importance of PTPs in physiological processes has been demonstrated in some cases. Cell line and germ line knockout experiments have shown that CD45 is essential for both T-cell signalling and the development of mature T-cells [7,8]. Haematopoietic cell PTP (*Hcph*) has been shown to have a vital role in haematopoiesis. Mice homozygous for the recessive allele motheaten (*me*) or viable motheaten (me^v) show multiple haematopoietic abnormalities, encompassing developmental and functional defects in macrophages, granulocytes, T-cells, B-cells and natural killer cells. These mutations lie within the *Hcph* gene and cause aberrant splicing of the *Hcph* transcript [9]. The characterization of *corkscrew* (*csw*), which is known to be maternally required for normal determination of cell fates at the termini of *Drosophila* embryos [10], showed it to be a member of the cytoplasmic family of PTPs [11]. Mutational analysis has shown that *csw* works in concert with D-*raf* to positively transduce the *torso* receptor protein-tyrosine kinase signal [11].

The overexpression of PTPα in rat embryo fibroblasts has been shown to result in persistent activation of c-*src* and transformation of the cells [12]. This suggests that PTPα may play a role in cell proliferation, mediating its action through the c-*src* signalling pathway. However, overexpression of PTPα in P19 embryonal carcinoma cells causes neuronal differentiation [13]. The cell type specificity of this response is likely to reflect differential responses to activated endogenous *src*.

To investigate the role of PTPs in relation to signalling in breast tumour cells, reverse transcription of RNA followed by PCR (RT-PCR) with degenerate primers was employed to characterize PTP-like molecules expressed in an involved (containing metastatic breast tumour) axillary lymph node [14]. The

Abbreviations used: PTP, protein-tyrosine phosphatase; rPTP, receptor-like PTP; GST, glutathione S-transferase; pNPP, *p*-nitrophenyl phosphate; RT-PCR, reverse transcription-PCR; EGFR, epidermal growth factor receptor; MAM domain, meprin–A5 antigen–PTPμ domain.

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The nucleotide sequence data reported have been submitted to the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under accession no. X95712.

present study reports the cloning and characterization of a novel rPTP identified by this strategy.

MATERIALS AND METHODS

RT-PCR methods

Total RNA was extracted from an involved (containing metastatic breast tumour tissue) axillary lymph node [14]. RNA was treated with DNase I (Pharmacia) and reverse-transcribed with random hexamer primers (Pharmacia) using avian myeloblastosis virus reverse transcriptase (Life Sciences). cDNA was amplified by PCR using degenerate oligonucleotide sense and antisense primers corresponding to the amino acid sequences (H/D)-FWRM(I/V)W and WPD(F/H)GVP respectively [15]. The amplifications were carried out in buffer containing 15 mM Tris/HCl (pH 8.8), 60 mM KCl, 2.25 mM $MgCl_2$, 0.2 mM of each dNTP and 2.5 units of *Taq* DNA polymerase (BRL). PCR cycling conditions were 94 °C for 1 min, 50 °C for 2 min and 72 °C for 1 min for a total of 30 cycles [15]. Fragments were subcloned into the PCRII plasmid (Invitrogen) to create a plasmid mini-library.

Isolation of cDNA clones

An amplified cDNA library constructed in λZAP from human foetal brain (Stratagene) was screened using the ^{32}P-radiolabelled PTP37 PCR fragment. Hybridization was carried out overnight at 65 °C in 7 % (w/v) SDS/0.25 M Na_2HPO_4 [16]. Filters were washed at a final stringency of 0.1 × SSC, 1 % (w/v) SDS, 0.1 % (w/v) $Na_4P_2O_7$ at 65 °C and subjected to autoradiography. A single clone (FB1-3) was converted into a phagemid vector by an *in vivo* excision protocol using the helper phage R408 (Stratagene). This clone was subsequently found to be truncated at the 5′ end. The clone (FB1-3) was digested with *Pst*I and the resulting fragments subcloned into pBluescript (Stratagene). The 5′-most subclone was then used to screen an MCF-7 human breast tumour cell line cDNA library constructed in λZAP [14]. This yielded a single cDNA containing an uninterrupted open reading frame. Clones were sequenced fully using both an automated sequencer (ABI 373A, Prism Ready Reaction Cycle Sequencing kit; Perkin Elmer) and single-stranded manual sequencing (Sequenase v2.0; USB).

In vitro translation

An *in vitro* transcription/translation kit (TNT® Coupled Reticulocyte Lysate System; Promega) was used to translate the full-length MCF-7 PTPπ clone. A pBluescript SK$^-$ (Stratagene) plasmid construct containing a fragment of the MCF-7 clone lacking all but 11 bp of the 3′ untranslated sequence was also translated using identical conditions. Transcription/translation were carried out on 1 μg of plasmid according to the manufacturer's instructions. A positive control construct containing the firefly luciferase gene was also translated.

Expression of PTPπ

A multi-tissue Northern (MTN) blot containing 2 μg of poly(A)$^+$ RNA from a variety of human tissues (Clontech) was hybridized with a ^{32}P-radiolabelled 1.1 kbp fragment encoding part of the extracellular domain. The filter was hybridized and washed according to the manufacturer's instructions and subjected to autoradiography. The membrane was also probed under the conditions described above with a fragment from the gene encoding β-actin, to analyse the integrity of the RNA.

Construction of glutathione S-transferase (GST) fusion expression vectors

Bacterial vectors expressing GST fusion proteins with the N-terminal (from foetal brain and MCF-7; residues 876–1252), C-terminal (residues 1083–1442), or both phosphatase domains in tandem (residues 876–1442) were constructed using pGEX 4T-1 (N-terminal and tandem catalytic domains) or pGEX 4T-3 (C-terminal catalytic domain) (Pharmacia).

Induction and purification of fusion proteins

Fusion protein expression was induced in 100 ml of exponentially growing cultures of *Escherichia coli* XL1-Blue (Stratagene) by adding 1 mM isopropyl β-D-thiogalactoside for 90 min at 37 °C. Proteins were purified from bacteria according to previously published methods [17], except that an additional pre-sonication freeze–thaw step was added to aid lysis.

Phosphatase assays using an artificial substrate

The phosphatase activity of GST fusion proteins bound to glutathione–Sepharose 4B was measured colorimetrically at A_{410} over several time points using the artificial substrate *p*-nitrophenyl phosphate (pNPP; Pharmacia) [18]. Samples of 25 μg of each protein, as assayed by the Coomassie Brilliant Blue dye-binding method [19], were used for the assays. A construct expressing the catalytic domain of human PTPβ fused to GST was used as a positive control. An *E. coli*-expressed GST (alone) was also assayed to determine the background phosphatase activity.

The amount of product formed after 10 min was calculated on the basis of a molar absorption coefficient for *p*-nitrophenol of $1.78 \times 10^4\ M^{-1} \cdot cm^{-1}$.

Purification of the epidermal growth factor receptor (EGFR) from A431 cells by immunoprecipitation

Approx. 5×10^6 cells were lysed in 1 ml of buffer comprising 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 % (v/v) Triton X-100 and 10 % (v/v) glycerol. Aprotinin (10 μg/ml), chymostatin (5 μg/ml), pepstatin A (5 μg/ml), antipain (5 μg/ml), leupeptin (10 μg/ml) and PMSF (2 mM final concentration) were added to the lysis buffer immediately before use. Lysates were incubated at 4 °C for 30 min. The lysed cells were centrifuged at 4 °C and the cleared lysate added to a complex comprising 60 μg of a rat-derived anti-EGFR antibody (ICR 62) attached to 500 μl of Protein A–Sepharose beads (Pharmacia) via a rabbit-derived anti-rat bridging antibody (Sigma).

In vitro autophosphorylation of the EGFR

Purified EGFR was incubated with 10 ng/ml EGF for 30 min at room temperature and labelled with 1 μCi of [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) in 500 μl of kinase buffer [50 mM Hepes, pH 7.0, 150 mM NaCl, 0.02 % (v/v) Triton X-100, 10 mM $MnCl_2$, 12 mM $MgCl_2$ and 10 % (v/v) glycerol].

Phosphatase assays using autophosphorylated EGFR as a substrate

The GST fusion protein containing the tandem catalytic domains of PTPπ (see Figure 5, lane 6) was purified on to glutathione–Sepharose beads as described above, and approx. 10 μg was added to the labelled EGFR–Sepharose complex in 100 μl of phosphatase assay buffer (20 mM Hepes, pH 7.0, 5 mM dithiothreitol). At several time points, aliquots were removed and

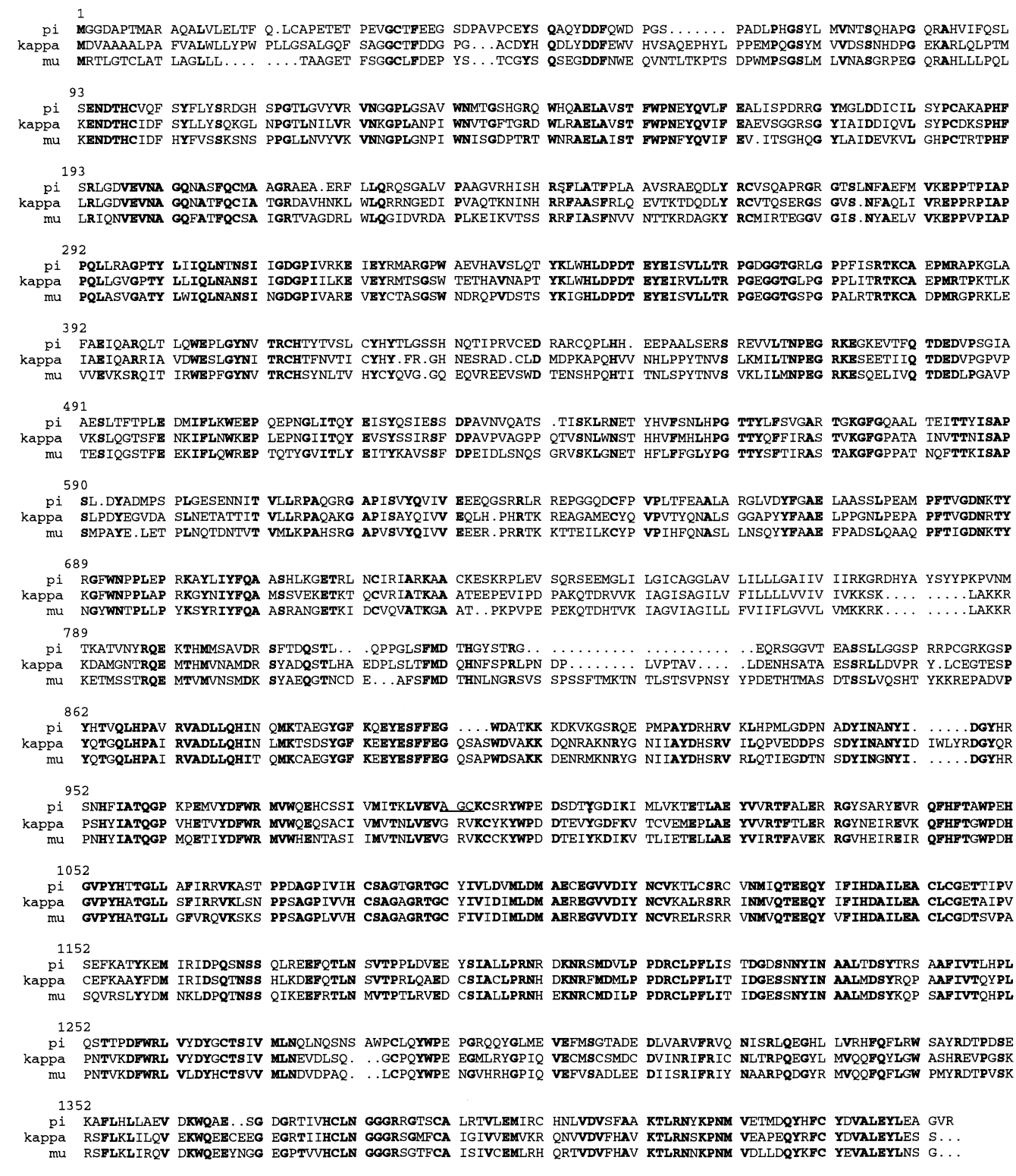

Figure 1 Alignment of PTPπ with the homophilic binding rPTPs, PTPμ and PTPκ

Residues conserved between all three sequences are depicted in bold. Underlined residues (PTPπ residues 991–993) represent the amino acids that are lacking in the foetal brain clone FB1-3. The various domains in PTPπ are: MAM domain (residues 33–183), IgG-like domain (209–263), fibronectin III repeats (291–675), transmembrane segment (749–770), N-terminal catalytic domain (913–1141) and C-terminal catalytic domain (1203–1438). The alignment was completed using PILEUP (GCG).

centrifuged to pellet the Sepharose beads. The resulting supernatants were counted in a scintillation counter for ^{32}P-labelled phosphate. A fusion protein containing the human PTPβ catalytic domain (10 μg) was used as a positive control, and *E. coli*-expressed GST alone (10 μg) was used as a negative control, to correct for background phosphatase activity.

RESULTS

Isolation and analysis of cDNA clones encoding a novel protein with the features of a rPTP

In order to identify novel PTPs expressed in metastatic breast tumours, reverse transcription followed by PCR using degenerate oligonucleotide primers was used to amplify cDNA fragments of approx. 250 bp encoding potential PTP catalytic domains. Plasmids were sequenced, and the novel sequence PTP37 was found not to be in the GenBank or EMBL nucleotide sequence databases (updated 19/01/95). After initial identification of PTP37, screening of a human foetal brain cDNA library yielded a 5 kb cDNA fragment which did not contain a full open reading frame. An approx. 6.3 kb clone subsequently isolated from a cDNA library derived from the MCF-7 human breast tumour cell line contained both putative initiation and termination codons. The cDNA, named PTPπ, encodes a predicted protein of 1442 amino acid residues (Figure 1) with the features of an rPTP. The putative start codon is identified by a consensus sequence for the initiation of translation [20], and is followed by sequences encoding a predicted hydrophobic leader sequence. The extracellular domain comprises a putative MAM (meprin–A5 antigen–PTPμ) domain, an IgG-like domain and four fibronectin III-like domains. Following a predicted transmembrane domain of 22 hydrophobic residues, two phosphatase-like catalytic units comprise the intracellular portion of the protein (Figure 1). In its primary sequence and predicted domain structure, PTPπ closely resembles two type II rPTPs, PTPμ and PTPκ, that have been shown to bind homophilically and to facilitate calcium-independent cell–cell adhesion [21–23]. It is likely that PTPπ represents a third member of this subfamily of type II rPTPs (Figures 1 and 2).

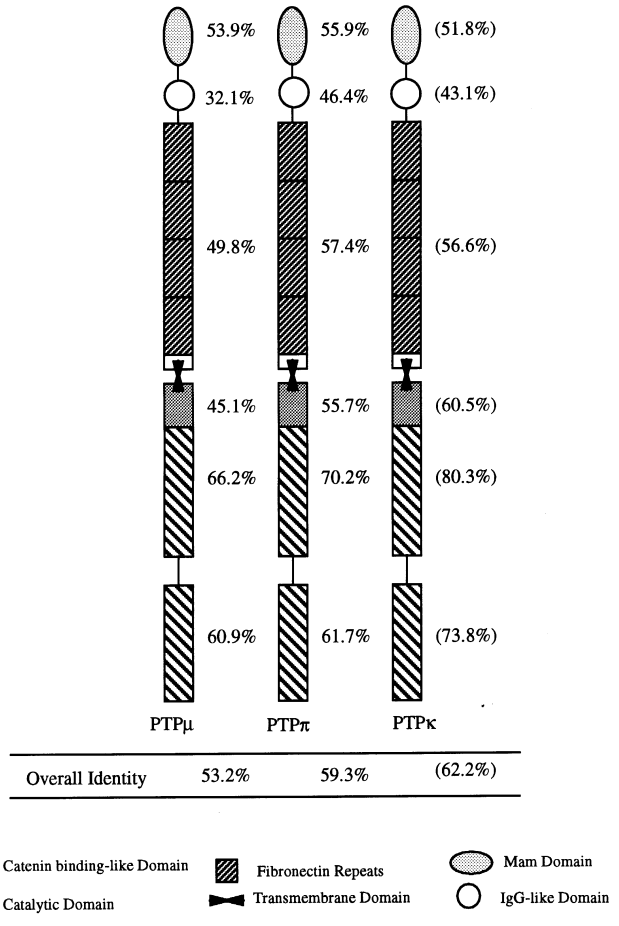

Figure 2 Schematic representations of PTPπ, PTPμ and PTPκ

Values represent the percentage identity of the indicated domains for PTPμ and PTPπ (left-hand column of numbers) and for PTPπ and PTPκ (centre column of numbers). The right-hand column of numbers, in parentheses, represents the percentage identities of the domains between PTPμ and PTPκ.

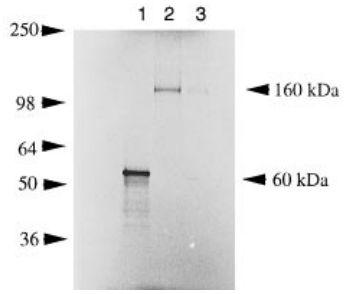

Figure 3 *In vitro* translation and SDS/PAGE analysis of PTPπ

Lane 1 shows the protein product of the firefly luciferase gene (60 kDa), used as a positive control. Lane 2 shows the protein product (160 kDa) of the MCF-7 breast tumour cell line-derived PTPπ clone that had been truncated to remove all but 11 bp of the 3′ untranslated sequence. Lane 3 shows the protein product (160 kDa) of the entire MCF-7 PTPπ clone. Values on the left are molecular masses of protein markers in kDa.

The human foetal brain-derived clone has a deletion of 9 bp within the sequence encoding the N-terminal phosphatase domain (Figure 1). *In vitro* translation of plasmid vectors (pBluescript SK$^-$; Stratagene) containing either the longest MCF-7 PTPπ clone, or a construct truncated at the 3′ end to exclude the all but 11 bp of the untranslated sequence, produced proteins of identical size which corresponded to the estimated size of approx. 160 kDa (Figure 3).

Expression of PTPπ

Northern blot analysis revealed a PTPπ transcript of approx. 6.3 kb present in the breast tumour cell lines MCF-7 and T47D (results not shown). Hybridization to mRNA purified from normal human tissues revealed that this PTPπ transcript was present in a variety of tissues, including skeletal muscle, heart, brain, pancreas, placenta, lung, liver and kidney. An approx. 2.4 kb transcript was also detectable in both heart and skeletal muscle (Figure 4).

RT-PCR analysis of sequences encoding part of the PTPπ N-terminal phosphatase domain revealed expression of the 9 bp longer form in human foetal brain, and in normal and malignant human breast tissue. A low level of expression of the shorter form was detected in foetal brain, but not in MCF-7 cells (results not shown).

PTPπ has two active catalytic domains

Constructs encoding GST fusion proteins containing each N-terminal domain alone, the C-terminal domain alone, or both domains (derived from the MCF-7 clone) in tandem gave products of $\sim$ 80 kDa (N-terminal catalytic domains), $\sim$ 80 kDa (C-terminal catalytic domain) and $\sim$ 95 kDa (tandem catalytic domains) in bacteria (Figure 5) (note that the C-terminal catalytic domain fusion protein is prone to proteolysis; however, significant measurable amounts of full-length product were puri-

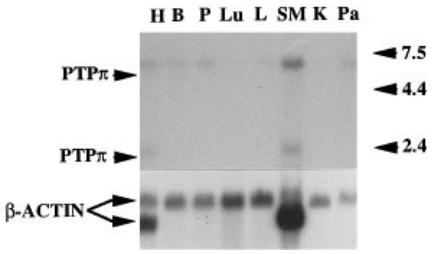

Figure 4 Northern analysis of the expression of PTPπ in human tissues

Arrowheads indicate size in kb. Lanes contain 2 μg of poly(A)$^+$ RNA from the following tissues: heart (H), brain (B), placenta (P), lung (Lu), liver (L), skeletal muscle (SM), kidney (K) and pancreas (Pa). The lower panel shows the same membrane probed with a β-actin cDNA.

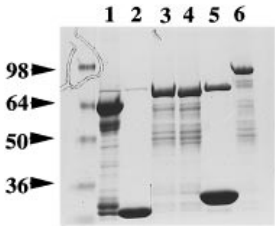

Figure 5 Coomassie Blue-stained gel showing Sepharose-purified PTPπ–GST fusion protein products

Markers are indicated in kDa on the left-hand side of the gel. Lane 1, PTPβ; lane 2, GST alone (pGEX 4-T1); lane 3, MCF-7 N-terminal catalytic domain (residues 876–1252) (pGEX N-Term); lane 4, foetal brain N-terminal catalytic domain (residues 876–1252) (pGEX delN); lane 5, C-terminal catalytic domain (residues 1083–1442) (pGEX C-Term); lane 6, both domains expressed in tandem (residues 876–1442) (pGEX Tandem).

Table 1 Phosphatase activities of PTPπ domains

The results show the accumulation of the product *p*-nitrophenol (measured as a colorimetric change) per μg of the various GST–PTP fusion proteins after a 10 min incubation with 10 mM pNPP. For details of fusion protein nomenclature, see the legend to Figure 5.

Fusion protein	Activity (pmol/μg)
pGEX 4-T1	0.048
N-Term	0.48
delN	0.42
C-Term	0.32
Tandem	1.08

fied). Each protein was shown to have inherent phosphatase activity, detected colorimetrically using the artificial substrate pNPP. Table 1 shows the average rate of metabolism of pNPP by each fusion protein over 10 min (data collected from three independent experiments).

Autophosphorylated EGFR is a substrate for PTPπ *in vitro*

The GST fusion protein containing the tandem catalytic domains (Figure 5, lane 6) was shown to have inherent protein phosphatase activity against autophosphorylated EGFR, as determined by release of phosphate from the immunoprecipitated protein. The immunoprecipitated protein was confirmed as EGFR by Western blotting (results not shown). Table 2 shows the release of free phosphate into the supernatant by the protein-phosphatase activity of PTPπ and PTPβ (data collected from two independent experiments).

Table 2 Protein-tyrosine phosphatase activity of PTPπ

Results show the release of ^{32}P-labelled phosphate (as percentage dephosphorylation) from autophosphorylated EGFR by fusion proteins comprising either the PTPπ tandem catalytic domains (pGEX Tandem) or the intracellular portion of PTPβ. GST alone (pGEX 4-T1) was included as a control for non-specifically associated phosphatase activity.

	Dephosphorylation (%)		
Time (min)	pGEX 4-T1	pGEX Tandem	PTPβ
1	0.005	0.2	6.8
5	0.02	1.5	19.8
10	0.02	14.6	67.9

DISCUSSION

This study reports the cloning and characterization of novel cDNAs encoding a human rPTP, PTPπ. The amino acid sequence inferred from the nucleotide sequence encodes two possible translation initiation methionine residues (designated amino acid residues 1 and 8). Both of these AUG codons are surrounded by good consensus sequences for the initiation of translation [20]. Upstream of the most 5′ AUG, the open reading frame continues for a further 60 nucleotides. To test the ability of one or both of these potential initiation codons to initiate translation, an *in vitro* rabbit reticulocyte transcription translation assay was used (see Figure 3). The resulting protein of $\sim$ 160 kDa confirmed that at least one of these codons was sufficient for the initiation of translation. Detailed studies on initiation codon usage indicate that the presence of a strong context around the first AUG codon usually precludes access to a second [20], and it is on this basis that we designate nucleotides 1–3 as the putative initiation codon. Experiments mutating each AUG codon independently could determine whether both are capable of initiating translation successfully. From amino acid residue 1, the N-terminal sequence of PTPπ bears most similarity to that of PTPμ, whereas if residue 8 were taken to be the initiation codon, more similarity is shown to PTPκ (see Figure 1).

PTPπ exhibits significant sequence similarity to two type II rPTPs, PTPμ [24] and PTPκ [25], which contain fibronectin III-like repeats, an IgG domain and a 160-residue N-terminal domain with similarity to the *Xenopus* A5 protein, known as the MAM domain (see Figures 1 and 2). Figure 2 gives percentage identity values over the various domains for PTPπ, PTPμ and PTPκ. From this it can be seen that, in general, PTPπ is more similar to PTPκ than to PTPμ when comparing extracellular domains, but the catalytic domains of PTPκ and PTPμ are more similar to each other than to that of PTPπ. PTPπ has a widespread expression pattern (Figure 4) and is present at detectable levels in various normal human tissues. PTPμ and PTPκ have been demonstrated to bind homophilically via their extracellular domains and to facilitate cell adhesion in non-adherent insect cells [21–23]. The individual contributions of the fibronectin III-like, MAM and IgG domains towards homophilic interactions are, however, unclear. Studies using proteins bound to beads indicated that the IgG domain alone of PTPμ was sufficient for binding; however, the MAM domain of PTPκ has been demonstrated to be a necessary component of the interaction, albeit in a different assay system [26,27]. The similarity of the extracellular domains of PTPμ, PTPκ and PTPπ raises the possibility of

heterophilic interactions. However, PTPμ and PTPκ will not interact with each other heterophilically [27]. Furthermore, a chimaeric protein in which the MAM domain of PTPμ is replaced with that of PTPκ, although capable itself of homophilic binding, will not bind to either PTPμ or PTPκ [27]. The fact that the distributions of PTPμ and PTPκ barely overlap [24,25] suggests that, even if heterophilic interactions were possible, they would not occur often *in vivo*. By analogy with PTPμ and PTPκ, the extracellular domain of PTPπ is likely to mediate homophilic binding; this possibility is currently under investigation.

PTPπ, in common with PTPμ and PTPκ, contains an extended sequence between the transmembrane domain and the first PTP catalytic domain. This has been shown to have identity with the catenin binding domain of cadherins [28]. Cadherins provide sites of association for the cytoskeleton with the plasma membrane at regions of cell adhesion. They are surface glycoproteins of 120–140 kDa comprising a large extracellular domain, a transmembrane region and, with the exception of T-cadherins [29], a cytoplasmic tail consisting of a catenin binding domain [30]. The catenins are cadherin-associated cytoplasmic polypeptides that are required for cadherin-mediated cell adhesion. At present there are three recognized mammalian catenin-like proteins: α-catenin, β-catenin and plakoglobin (γ-catenin). Deletion of the catenin binding site from E-cadherin abrogates cadherin-dependent cell adhesion [31], and non-adhesive epithelial tumour cells lacking α-catenin can be induced to form tightly adherent epithelia when transfected with α-catenin. It is thought that α-catenin, via a β-catenin-mediated association with cadherins, forms a necessary link between cadherins and the actin cytoskeleton [32]. The β-catenin–E-cadherin complex has been shown to be a substrate for v-*src* in transformed epithelial cells, and the phosphorylation state of this complex has been shown to affect the differentiation and the invasiveness of these cells [33]. Experiments with PTPμ have shown that it associates with the E-cadherin–α/β-catenin complex, and it has been postulated that E-cadherin may be a substrate for PTPμ [28]. Furthermore, PTPμ has been shown by immunocytochemistry to gather at cell junctions [34]. This evidence may indicate important roles for PTPμ, PTPκ and PTPπ in cell–cell interactions, both directly via homophilic binding and indirectly via their association with the cadherin–catenin complex.

Using the artificial substrate pNPP, phosphatase activity has been detected in both the N-terminal and C-terminal catalytic domains of PTPπ. The N-terminal domain encoded by the foetal brain-derived cDNA fragment, which shows a deletion of three amino acids compared with the breast tumour-derived N-terminal domain, was shown to have catalytic activity (Table 1). It is not known where the phosphatase activities of PTPμ and PTPκ reside, and whether both N- and C-terminal domains are active. However, it can be seen from Figure 1 that the three extra residues in the breast tumour-derived N-terminal domain have corresponding residues present in the C-terminal domain of PTPπ. Furthermore, these residues have cognates in the N-terminal phosphatase domains of both PTPμ and PTPκ, but are absent from their C-terminal domains. Further experiments could clarify whether these residues are important for the regulation of catalytic activity in either domain; this may require the determination of natural substrates for PTPπ.

PTPπ has protein phosphatase activity, as demonstrated with autophosphorylated EGFR *in vitro*. Using the purified GST fusion protein containing the two catalytic domains in tandem, an *in vitro* assay showed release of free phosphate from autophosphorylated EGFR. Concurring with results using the artificial substrate pNPP (not shown), PTPπ showed less activity against the substrate than did the positive control, PTPβ. Whether this is due to the incorrect folding of the PTPπ catalytic domains, a lack of appropriate cofactors/protein modifications, or substrate specificity is unclear. Such possibilities could be the subject of further investigations.

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