RESEARCH COMMUNICATION

TPIP: a novel phosphoinositide 3-phosphatase

Steven M. WALKER, C. Peter DOWNES and Nick R. LESLIE¹

Division of Cell Signalling, School of Life Sciences, University of Dundee, MSI/WTB Complex, Dow Street, Dundee DD1 5EH, Scotland, U.K.

The PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumour suppressor is a phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5) P_3] 3-phosphatase that plays a critical role in regulating many cellular processes by antagonizing the phosphoinositide 3-kinase signalling pathway. We have identified and characterized two human homologues of PTEN, which differ with respect to their subcellular localization and lipid phosphatase activities. The previously cloned, but uncharacterized, TPTE (transmembrane phosphatase with tensin homology) is localized to the plasma membrane, but lacks detectable phosphoinositide 3-phosphatase activity. TPIP (TPTE and PTEN homologous inositol lipid phosphatase) is a novel phosphatase that occurs in several differentially spliced forms of which two, TPIP α and TPIP β , appear to be functionally distinct. TPIP α displays similar phosphoinositide 3-phosphatase activity compared with PTEN against PtdIns(3,4,5) P_3 , PtdIns(3,5) P_2 ,

PtdIns(3,4) P_2 and PtdIns(3) P , has N-terminal transmembrane domains and appears to be localized on the endoplasmic reticulum. This is unusual as most signalling-lipid-metabolizing enzymes are not integral membrane proteins. TPIP β , however, lacks detectable phosphatase activity and is cytosolic. TPIP has a wider tissue distribution than the testis-specific TPTE, with specific splice variants being expressed in testis, brain and stomach. TPTE and TPIP do not appear to be functional orthologues of the Golgi-localized and more distantly related murine PTEN2. We suggest that TPIP α plays a role in regulating phosphoinositide signalling on the endoplasmic reticulum, and might also represent a tumour suppressor and functional homologue of PTEN in some tissues.

Key words: C2 domain, endoplasmic reticulum, inositol lipid, PTEN, TPTE.

INTRODUCTION

The PTEN (phosphatase and tensin homologue deleted on chromosome 10) lipid phosphatase is a tumour suppressor that is mutated in a wide range of sporadic human tumour types. Germ-line mutation of PTEN is also associated with the human conditions Cowden disease and Bannayan–Riley–Ruvalcaba syndrome, which are characterized by multiple benign growths and an increased risk of malignancies, including breast and thyroid tumours. In agreement with this tumour suppressor status, PTEN activity in many cell types appears to inhibit cell cycle progression, cell survival and cell motility (reviewed in [1–4]).

PTEN is a lipid phosphatase with specificity for phosphate on the 3 position of the inositol ring of a number of phosphoinositide signalling molecules [5,6]. However, the best characterized cellular substrate of PTEN is phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5) P_3], and PTEN is a direct antagonist of growth-factor-stimulated phosphoinositide 3-kinases (PI 3-kinases) [1–3,7]. It appears that much of the tumour suppressor function of PTEN is mediated through controlling the activity of the PtdIns(3,4,5) P_3 -regulated kinase, protein kinase B (PKB, also known as Akt). However, other signalling pathways are also clearly regulated by PTEN, and their significance in tumour development is becoming more clear [8–10]. The determination of the crystal structure of PTEN not only revealed the structure of the N-terminal phosphatase domain, but also identified a tightly associated C-terminal C2 domain [11]. Significant deletion and mutagenesis studies indicate that both the phosphatase domain and C2 domain form a minimal catalytic unit, as even small deletions of the C2 domain remove detectable phosphatase

activity against soluble substrates [11–14]. Evidence suggests that the role of the C2 domain may be to correctly orientate the active site of the enzyme for catalysis on membranes, and possibly to mediate transient membrane association of this largely cytosolic protein [2,11,15].

Recently, phosphatases closely related to PTEN have been described by two research groups [16,17]. A testis-specific gene product, TPTE (transmembrane phosphatase with tensin homology), was cloned in 1999 [16], although the protein has not been characterized. More recently, a sequence presented as the murine homologue of TPTE (mPTEN2) is expressed specifically in the testis and encodes an active 3-phosphoinositide phosphatase localized on the Golgi apparatus [17].

In the present study, through analysis of PTEN-like sequences in the draft human genome, we have identified and characterized a new PtdIns(3,4,5) P_3 3-phosphatase, TPIP (TPTE and PTEN homologous inositol lipid phosphatase), with homology to PTEN within the phosphatase and C2 domains. We also show that TPIP has phosphoinositide phosphatase activity, unlike the closely related TPTE, and that both TPIP and TPTE are found in different parts of the cell from the more distantly related mPTEN2. Therefore these three proteins are likely to represent distinct members of a PTEN-related family.

EXPERIMENTAL

Cloning and plasmid construction

PCR products encoding the phosphatase domain and C2 domain of TPIP were cloned from a human testis library (ClonTech), using the primers YS1 (5'-GGATCCAGATGGACACATTTA-GTTCG-3') and YA2 (5'-TGTCATTGGAAGTCATTTC-

Abbreviations used: ER, endoplasmic reticulum; GFP, green fluorescent protein; PDI, protein disulphide isomerase; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TPTE, transmembrane phosphatase with tensin homology; mPTEN2, murine homologue of PTEN; TPIP, TPTE and PTEN homologous inositol lipid phosphatase.

¹ To whom correspondence should be addressed (e-mail n.r.leslie@dundee.ac.uk).

TTG-3'), and then cloned into the vector pCR2.1 TOPO (Invitrogen) before sequencing. This gave two predominant splice variants of this TPIP cDNA fragment (1 and 2), which were then subcloned as *EcoRI*–*Bam*HI fragments into these sites of pGEX-6P1 (Amersham Pharmacia Biotech) and fully re-sequenced. To identify the 5' end of the TPIP cDNA, nested PCR was employed, and each round used human testis cDNA as a template, a TPIP-specific reverse primer and a forward primer from within the library vector. The first round of PCR used a forward primer corresponding to the pGAD cDNA library vector, 5'-CACTA-CAGGGATGTTTAATACC-3', and a reverse outer primer from TPIP, 5'-TAGTCCCGGTTCTTCCTTTG-3'. A 1 in 10 dilution of the products generated from this PCR was used as a template for a second round, using a second forward primer corresponding to vector sequences and another reverse primer corresponding to TPIP, 5'-CGGCAGTTAAAAATATTTTCGG-3'. This generated a single PCR product that was sequenced. PCR and cloning (in pCR2.1TOPO) of several full-length coding cDNAs using a 5' primer (5'-AGGGATCCACTAGTATGAATGAAAGTC-3'), introduced restriction sites allowing subsequent subcloning. The full-length coding TPTE β cDNA was cloned using PCR from a human testis cDNA library using the primers 5'-CGTGGCAC-ATATGAATGAAAGTCCTGATCCG-3' and 5'-GGCAGGA-TCCTTAATCGGATCCAGCTACAAC-3'.

The full-length coding sequences of TPIP α and TPIP β were subcloned into pEGFP-C1 (ClonTech) using a *Bam*HI–*Eco*RI digest of these pCR2.1TOPO TPIP constructs and a *Bgl*II–*Eco*RI digest of the vector, and this was subsequently sequenced. The TPTE β cDNA was cloned into pEGFP-C1 and pEGFP-N3 to give expression vectors with the green fluorescent protein (GFP) tag being N- and C-terminal respectively. These two GFP–TPTE fusion proteins appeared to have similar localizations and activities in functional assays. The GFP–PTEN expression vector has been described previously [18].

Phosphatase-dead constructs were made by QuikChange mutagenesis (Stratagene) of the catalytic cysteine residue (Cys²⁴³ of TPIP α and Cys³⁰⁰ of TPTE β) to a serine using the primers C243S-S (5'-CGTAGCGATTCACAGTAAAGGAGGCAA-AG-3') and C243S-AS (5'-CTTTGCCTCCTTTACTGTGAATCGCTACG-3'), and C300S-S (5'-GAAAACATCGTAGCGATTCACAGTAAAGGAGGCACAGATAG-3') and C300S-AS (5'-CTATCTGTGCCTCCTTTACTGTGAATCGCTACGATGTTTC-3'). Each coding sequence was then re-sequenced.

Bioinformatics

The prediction of the secondary and three-dimensional structures was performed using PsiPred and Swiss-Model respectively. Both were accessed through the ExPASy website, run by the Swiss Institute of Bioinformatics. PsiPred is from Brunel Bioinformatics Group, Department of Biological Sciences, Brunel University, U.K. Transmembrane domains and topology were predicted using several programmes, particularly TMHMM from the Technical University of Denmark, Lyngby, Denmark [19]. Multiple sequence alignments were performed using ClustalW and BoxShade, both run by EMBnet, Lausanne, Switzerland.

Expression and purification of TPIP and TPTE

Fragments of TPIP and TPTE encompassing the phosphatase domain and C2 domain were expressed and purified from bacteria as glutathione S-transferase fusion proteins. TPIP fragments 1 and 2 correspond to the C-termini of TPIP α (residues 100–445) and TPIP β (residues 46–326) respectively. The TPTE fragment corresponds to residues 204–551 (numbering from TPTE α). Expression plasmids based on pGEX-6P1 were transformed

into *Escherichia coli* strain BL21, and induced at 26 °C overnight with 50 μ M isopropyl β -D-thiogalactoside. Expressed glutathione S-transferase fusion proteins were then purified according to the manufacturer's instructions using GSH–Sepharose 4B (Amersham Pharmacia Biotech).

Tissue distribution

The tissue distribution of TPIP expression was determined by PCR from human tissue cDNA samples (human cDNA panel, ClonTech, and stomach cDNA, Ambion) and human cDNA libraries (testis and liver were from ClonTech, brain and skeletal muscle were gifts from Dr Peter Cheung and Dr Margaret Lawlor respectively, both of the MRC Protein Phosphorylation Unit, Wellcome Trust Biocentre, Dundee, Scotland, U.K.). Actin-specific and a number of TPIP-specific primers were used, as well as primers designed to give a product from TPIP, TPTE and other related phosphatases. The TPIP PCR (see Figure 2A) used the primers YS5', 5'-TATTCTGATAAGAATTTTTCATCTGC-3', and YS3', 5'-CCTCGGCAGTTAAAAATATTTTCG-3'. The TPTE PCR (see Figure 2B) used primers to specifically amplify TPTE α (5'-AACCCTATCGAGTCTACAATC-3' and 5'-GCACCTTTCACATCATCATACAGAGGTGG-3') and TPTE β (5'-CTGGTGTACCTATCAGTAAAA-3' and 5'-GAGGGGAGATTCCAGTTGTAG-3'). PCR was carried out using a HiFidelity Expand Kit (Roche), and samples from spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leucocyte, brain, skeletal muscle, liver and stomach. Products from all tissues were cloned using the pCR2.1 TOPO cloning vector (Invitrogen) and then sequenced.

Cellular localization

Cells expressing GFP-fusion proteins were observed and photographed using a Leica inverted stage fluorescence microscope with a Hamamatsu Orca charge-coupled-device camera. Images were analysed using Improvision OpenLab deconvolution software. Cells were fixed in 3% (w/v) paraformaldehyde/PBS, and immunofluorescent labelling followed standard protocols with cells being made permeable with 0.2% Triton X-100/PBS and using fish skin gelatin (Sigma) as a blocking agent. Antibodies for immunofluorescence were kindly provided by J. Lucocq (School of Biological Sciences, University of Dundee, Dundee, Scotland, U.K.), S. Ponnambalam (School of Biochemistry and Molecular Biology, University of Leeds, Leeds, U.K.) and M. Lowe (School of Biological Sciences, University of Manchester, Manchester, U.K.).

Phosphatase assays

Radiolabelled substrates were made by phosphorylating unlabelled inositol lipids (Cell Signals, Lexington, KY, U.S.A.) in the 3 position using purified recombinant PI 3-kinase γ . Substrates were presented at a mole fraction of 5% of phosphatidylcholine vesicles prepared by sonication. Phosphoinositide phosphatase assays were performed in an assay buffer containing 10 mM Hepes, pH 7.0, 150 mM NaCl, 2 mM EGTA and 10 mM dithiothreitol. Recombinant TPIP fragments 1 and 2, and TPTE, were assayed for their ability to release [³³P]_i from radiolabelled PtdInsPs. Release of radiolabelled phosphate was determined by performing a modified Bligh and Dyer extraction [20]. The upper phase containing P_i was removed, dried and resuspended in a 1 M trichloroacetic acid/1% molybdate solution. After extraction with 2 vol. of toluene/isobutyl alcohol (1:1, v/v), the upper phase was removed and the amount of radioactivity was measured.

RESULTS

Cloning of TPPI

Analysis of the draft human genome revealed at least seven apparent gene sequences with significant homology to the PTEN phosphatase domain, in addition to the PTEN gene at chromosome 10q23 and a PTEN pseudogene at chromosome 9p21. These seven sequences each have at least 14 apparent exons spread over greater than 60 kb and form a distinct family, being more closely related to each other than to PTEN or other more distant members of the protein tyrosine phosphatase superfamily. One of these genes, on chromosome 21, was identified in 1999 and named TPTE [16,21], but results concerning its activity, cellular localization or function have not been described. Other sequences are found on chromosomes 10 and Y, and at least four gene copies are located on chromosome 13.

cDNAs for three of these sequences were cloned by PCR from a human testis cDNA library, and corresponded to TPTE and two novel cDNAs, named ψ TPT and TPPI, both from chromosome 13. ψ TPT seems likely to be a transcribed unprocessed

pseudogene as the cDNA lacks extensive open reading frames and the PTEN/TPTE homologous reading frame is interrupted by a frameshift and an in-frame stop codon (results not shown). In contrast, partial cDNAs for TPPI were found to contain an extensive open reading frame with homology to the PTEN and TPTE genes. The 5' end of this cDNA was cloned by nested PCR using TPPI-specific primers and primers within the library vector, which yielded a single product corresponding to sequences contained within the TPPI gene. Further attempts have also yielded an additional sequence that appears to represent an alternative longer upstream untranslated region. Therefore it appears that translation of the predicted TPPI protein initiates at the methionine residue corresponding with that which initiates TPTE. The published TPTE cDNA sequence (GenBank® accession number AF007118) encode differed from our cloned TPTE cDNA sequence at the N-terminus of the encoded protein (hereafter TPTE α and TPTE β respectively). The cloned sequence had a small deletion in the N-terminal region before the first transmembrane domain. It is also of note that within the human population there appears to be a polymorphism near to the C-

Figure 1 TPPI: a novel PTEN-like phosphatase

(A) An alignment of the amino acid sequences of PTEN, TPPI α , TPPI β , TPTE α and mPTEN2 is shown, with identical residues being shaded in black and similar residues in grey. The predicted active-site P-loop sequence is boxed and the T1 loop is marked with a broad horizontal bar. (B) The domain structures of TPPI α , TPPI β , TPTE α , TPTE β and PTEN are shown. The predicted active-site and catalytic cysteine residues are represented within the phosphatase domain by a circled letter C. Non-conserved phosphorylation sites in the PTEN C-terminus are shown by a circled letter P. Amino acid numbering for domain boundaries is predicted from the PTEN crystal structure [11]. Predicted transmembrane spanning regions are shown by filled vertical boxes. TPPI α and TPPI β differ in two regions. One splicing event introduces 54 amino acids, including two membrane-spanning regions in the N-terminus of TPPI α . The other splicing event introduces an alternative exon and stop codon in the TPPI β mRNA, resulting in a truncated C2 domain. (C) The predicted membrane-spanning topology of TPPI α is shown as the predicted probability of membrane insertion along the amino acid sequence. This prediction was performed using the TMHMM programme [19].

terminus of TPTE, since several human expressed sequence tag sequences encode a Ser in place of the more common Tyr at position 482 (numbering from TPTE α). The predicted amino acid sequence of TPIP, and an alignment with TPTE, PTEN and mPTEN2, are shown in Figure 1(A).

The translated sequence of the TPIP cDNA (TPIP α) shows a 445-amino-acid open reading frame with a very high degree of sequence conservation compared with the TPTE protein. Both TPIP and TPTE share the most similarity with PTEN in their phosphatase domains and to a lesser extent with the PTEN C2 domain. Significantly, both are predicted to display similar secondary and three-dimensional structures to PTEN, even in the regions that lack a high level of sequence identity. TPIP α has two strong and one more weakly predicted N-terminal transmembrane domains that are absent from PTEN (Figure 1C), whereas TPTE has three. There are, however, some potentially significant differences between TPIP and TPTE, especially in the putative active site. The corresponding residues that make up the active-site pocket in PTEN are mostly conserved in the TPIP and TPTE proteins, with the exception of an alanine-to-glycine change in the P loop and a longer TI loop. This longer loop is not predicted to restrict access to the active-site pocket, and in TPTE has two additional acidic residues replacing asparagine residues in TPIP. In addition to these changes, TPTE also has adjacent lysine and glycine residues present in PTEN and TPIP (Lys¹²⁸ and Gly¹²⁹ in the P loop of PTEN) replaced with a threonine and an aspartic acid respectively. These non-conservative changes might well be expected to alter the basic character of the active-site pocket and affect substrate binding, particularly since when mutated both residues in PTEN have been shown to alter the relative activity of PTEN against different substrates [6,11,22]. Lys¹²⁸ in PTEN also appears to be positioned next to the phosphoinositide substrate D5 phosphate [11]. Both TPIP α and TPTE have significant C-terminal amino acid homology with the PTEN C2 domain (TPIP 30% amino acid identity, see Figure 1A). Predictions of the secondary and three-dimensional structures indicate a very close conservation of β strands and a C2-domain structure respectively. Both TPIP and TPTE lack a C-terminal tail beyond the putative C2 domain, which in PTEN contains a PDZ-binding domain and phosphorylation sites, and appears to regulate the stability and, possibly, functioning of the PTEN protein [1–3].

TPIP is expressed in several differentially spliced forms

Cloning of full-length coding cDNAs by PCR from a human testis cDNA library also revealed that TPIP is expressed as a number of differentially spliced forms. Two predominant forms are expressed in the testis, termed TPIP α and TPIP β . These are proteins of 445 and 326 amino acids respectively (Figure 1B). TPIP α and TPIP β differ at both the N- and C-termini, with TPIP α being predicted to contain several transmembrane spanning domains and a predicted C-terminal C2 domain. In contrast, TPIP β has no predicted transmembrane domains and an alternative exon that contains a stop codon and leads to truncation of the putative C2 domain. Due to the predominant expression of these isoforms, we have used these proteins in our functional characterization. However, several other splice-variants of TPIP are expressed in the testis and in other tissues (S. M. Walker, N. R. Leslie and C. P. Downes, unpublished work).

Tissue distribution

Analysis of the tissue distribution of TPIP gene expression was performed using PCR with human tissue cDNA samples. This showed that TPIP is highly expressed in the testis, as is TPTE.

Figure 2 Tissue distribution of TPIP

(A) PCR using human tissue cDNA as template was performed with primers specific for actin and for the phosphatase domain of TPIP. All TPIP PCR products were cloned and sequenced to verify their origin. (B) PCR was performed using reverse primers specific for TPIP α and TPIP β from within the region encoding the putative C2 domain, and forward primers common to both these sequences. Human tissue cDNA, and TPIP α and TPIP β plasmid controls were used as templates.

However, in contrast with TPTE, TPIP is also expressed in the brain and the stomach (Figure 2). Semi-quantitative PCR analysis suggests that TPIP is most highly expressed in the testis, at high levels in the brain and at lower levels in the stomach. Primers specific for TPIP α and TPIP β show that whereas TPIP β appears only to be expressed in the testis, TPIP mRNA species, including the sequences encoding the TPIP α C2 domain, are expressed in testis, brain and stomach (Figure 2B). However, size differences indicate that these are not identical to TPIP α , and will require further characterization. Northern blotting was not used to assess tissue distribution due to the extremely high degree of similarity between the TPIP sequence and other related genes (e.g. between TPIP and TPTE there is 94% nucleotide identity over the common coding regions, and 77% identity including the three differentially spliced exons) and the lack of unique sequences. Analysis of public nucleotide sequence databases revealed only one expressed sequence tag sequence from TPIP (GenBank® accession number AW393549) that was obtained from a human stomach cDNA library.

Cellular localization

The cellular localization of TPIP and TPTE investigated using proteins tagged with GFP. GFP-fusion proteins of TPIP α ,

Figure 3 Cellular localization of TPIP α , TPIP β , TPTE β and PTEN

(A–D) N-terminally GFP-tagged proteins were expressed in HEK293 cells for 24 h and revealed by fluorescence microscopy. (A) GFP–TPIP α , (B) GFP–TPIP β , (C) GFP–TPTE β and (D) GFP–PTEN. (E–H) COS7 cells were transfected with expression vectors for GFP–TPIP α and fixed after 24 h. The localization of GFP–TPIP α (E), endogenous PDI (F) and these merged images (G) are shown. (H) shows a merged image of GFP–TPIP α and the endogenous *trans*-Golgi protein TGN46 (red). In this image, cellular DNA is stained with 4,6-diamidino-2-phenylindole (blue). Some of the COS7 cells in these cultures display a diffuse pattern of Golgi staining that appears not to be related to expression of TPIP α .

TPIP β , TPTE β and PTEN were expressed in HEK293 and Swiss 3T3 cells, and the localization of these proteins observed using deconvolved fluorescence microscopy. These results show that GFP–TPIP β , which lacks a discernible membrane association motif, appears to be cytosolic. However, GFP–TPIP α and GFP–TPTE, which both contain apparent N-terminal membrane-spanning domains, display strong but distinct membrane localizations. GFP–TPIP α appears to be associated with intracellular membranes, whereas GFP–TPTE β is associated with the plasma membrane (Figure 3). Further experiments investigating the localization of TPIP α in COS7 cells found a close co-localization of GFP–TPIP α with protein disulphide isomerase (PDI) the (luminal) endoplasmic reticulum (ER) marker, but not with the *trans*-Golgi marker TGN46 (Figure 3).

TPIP is a phosphoinositide 3-phosphatase

In order to assay these putative phosphatases for enzymic activity, the phosphatase/C2-domain fragments of TPIP and TPTE lacking the N-terminal transmembrane domains were expressed

Figure 4 TPIP is a phosphoinositide 3-phosphatase

Assays were performed at 37 °C for 30 min. Recombinant protein (100 ng) was used in each assay. TPIP 1, TPIP 2 and TPTE correspond to the phosphatase domain and C2-domain-containing fragments of TPIP α (residues 100–445), TPIP β (residues 46–326) and TPTE (residues 204–551) respectively. TPIP C-S corresponds to TPIP fragment 1 with an inactivating mutation in the catalytic cysteine residue. Inositol phospholipid substrates were labelled in the 3 position with [32 P]P $_i$ and presented at a mole fraction of 5% of phosphatidylcholine vesicles prepared by sonication. Each data point represents the mean $\pm$ S.E.M. for six independent determinations from two experiments.

and purified from bacteria. Due to the differential splicing of TPIP α and TPIP β in the putative C2 domain, two phosphatase/C2 domain fragments were expressed; fragments 1 and 2 corresponding to these regions of TPIP α and TPIP β respectively. To control for co-purifying phosphatases, the putative active-site cysteine residues were mutated to serine residues (Cys 243 $\rightarrow$ Ser in full-length TPIP α) and wild-type proteins were purified alongside these phosphatase-dead mutants. These proteins were assayed against a range of inositol lipids labelled in the 3 position, and presented as vesicular substrates. TPIP fragment 1 was found to have good activity against each substrate, whereas the phosphatase-dead mutant of this fragment, the splice variant fragment 2 and TPTE did not display detectable activity (Figure 4). This shows that TPIP can act as a phosphoinositide

Table 1 TPIP dephosphorylates the 3 position of the inositol ring of PtdIns(3,4,5)P₃

PtdIns(3,4,5)P₃ labelled in the 3 position with [³³P]P_i was incubated with either the wild-type TPIP fragment 1 or with the phosphatase-dead mutant (TPIP C-S). P_i release was measured as described in the Experimental section. Lipid products were extracted, deacylated and analysed by HPLC as described previously [6,20]. ND, not detectable when 0.5% of the radioactivity in the starting substrate would have been detected if this amount had accumulated in the elution position expected for PtdIns(3,5)P₂ and PtdIns(3)P or PtdIns(3,4)P₂.

Substrate	Percentage composition of [³³ P]P _i label		
	Time zero	TPIP C-S	TPIP fragment 1
PtdIns(3,4,5)P ₃	97	96.3	68.1
PtdIns(3,4)P ₂	3	3	2.7
PtdIns(3,5)P ₂	ND	ND	ND
PtdIns(3)P	ND	ND	ND
P _i	ND	0.7	29.2

Figure 5 TPIP does not regulate PKB phosphorylation

An haemagglutinin (HA)–PKB co-transfection assay in PTEN-null U87MG cells was used as described previously [18]. Cells were co-transfected with HA–PKB and expression vectors for the indicated proteins fused to GFP. HA–PKB was immunoprecipitated, and phosphorylation was assessed using antibodies specific for phosphoserine at position 473 of PKB (pS-473 PKB). Abbreviations: IP, immunoprecipitation; WB, Western blot; wt, wild-type; C189S, Cys¹⁸⁹ → Ser; C243S, Cys²⁴³ → Ser.

3-phosphatase. In order to investigate whether TPIP has phosphatase activity that is directed only against the 3 position of the inositol ring, the reaction products after incubation of PtdIns(3,4,5)P₃ (labelled at the 3 position with [³³P]P_i) with TPIP fragment 1 were deacylated and analysed by HPLC. In these experiments, approx. 30% of the incorporated label was lost from the PtdIns(3,4,5)P₃ fraction and recovered quantitatively as released [³³P]P_i, with no other novel dephosphorylated species being detected (Table 1). Since other products were not detected and together with the activity results presented in Figure 4, these results indicate that the phosphatase activity of TPIP is specific for the 3 position of the inositol ring.

In addition to activity against inositol lipid substrates, PTEN also has activity *in vitro* against some soluble inositol phosphates [5,6,23]. Therefore TPIP (fragment 1) was assayed with Ins(1,3,4,5)P₄ labelled at the 3 position with [³³P]P_i using conditions similar to those described for the lipid substrates. In these experiments, in which PTEN was found to exhibit activity comparable with that described previously [5,6], no activity was detectable with TPIP fragment 1 (results not shown). The sensitivity of these experiments was such that the TPIP activity would have been detected had it been greater than approx. 10%

of that seen with PTEN. These results suggest that Ins(1,3,4,5)P₄ is not a substrate for TPIP.

Since PTEN is known to regulate the activity of PKB, we investigated the ability of TPIPα, TPIPβ and TPTEβ to regulate PKB. Using a tagged-PKB co-transfection assay in U87MG cells as described previously [18], PTEN caused a dramatic reduction in PKB phosphorylation, but TPIPα, TPIPβ or TPTEβ did not have any detectable effect when expressed at comparable levels (Figure 5).

DISCUSSION

We have identified a new homologue of PTEN, TPIP, which displays a distinct cellular localization, but similar phosphoinositide phosphatase activity, when compared with PTEN. Phosphatase activity was detected using TPIP fragment 1 that encompasses the phosphatase domain and C2 domain, but not fragment 2 that had only a partial C2 domain. Since the fragment 1 and 2 sequences came directly from the sequences of TPIPα and TPIPβ respectively, this indicates that membrane-associated TPIPα should have phosphatase activity, but the cytosolic TPIPβ may not. These results also strongly suggest that the putative C2-domain sequence of TPIPα is required for phosphatase activity, as is the case with PTEN, and that the C-terminus of TPIPα indeed forms a C2-domain structure. Significantly, using an almost identical expressed protein fragment from the closely related protein, TPTE, we failed to detect phosphatase activity against the 3 position phosphorylated lipid substrates used. This suggests that TPTE probably does not have the phosphoinositide phosphatase activity and/or specificity of TPIPα, possibly due to the significant alterations to the P loop of the active-site pocket and/or the TI loop. It is possible, of course, that TPTE has activity against other substrates, possibly the acidic protein substrates that PTEN can dephosphorylate [24]. Possible mechanisms for the function of expressed inactive phosphatase homologues have been proposed, including acting as targeting domains through substrate-like binding of the phosphatase domain, 'dominant negative' substrate traps and others [25].

While the present study was in progress, a report described the cloning of a novel PTEN homologue from mouse sequences, and named this PTEN2 [17]. The authors presented the sequences of human TPTE and a novel mouse sequence as orthologues, and revealed phosphoinositide phosphatase activity, testis-specific expression and a Golgi localization using the mouse protein [17]. However, it seems very unlikely, given the active-site sequence divergence, the apparent lack of activity and the plasma membrane localization of TPTE, that it is a functional homologue of mPTEN2. It is also unlikely that TPIP and mPTEN2 represent functional homologues, since the differences in cellular localization, number of predicted transmembrane domains and apparent tissue distribution, as well as the presence of several other uncharacterized genes in the human genome, argue against this possibility. It is also of note that the sequences of TPIP and TPTE are more highly conserved (85% identical through the phosphatase and C2 domains) than either protein is to the conserved regions of mPTEN2 (71% and 69% identical respectively).

TPIPα has two, or possibly three, putative transmembrane domains that are absent in TPIPβ. TPIPα appears to be found on the ER, as it co-localizes with PDI and has a reticular distribution, including some nuclear envelope association, which is a characteristic of ER proteins. These results would suggest that TPIPα is inserted into membranes of the ER, however, without experimental determination it is difficult to predict whether the

phosphatase domain of TP1P α is luminal or cytosolic. This localization would have significant implications for its function. Most signalling-lipid-metabolizing enzymes are not integral membrane proteins, but rather have transient and often regulated interactions with cellular membranes. Recent kinetic analysis and other results suggest that PTEN is a highly efficient lipid phosphatase that associates only transiently with cellular membranes (G. McConnachie and C. P. Downes, unpublished work, and [8,11]). Since the catalytic fragment of TP1P α displays broadly similar activity to PTEN, it seems likely that TP1P α activity would restrict its substrate lipids to extremely low levels in the membrane compartments on which it is found. Whereas PtdIns(3,4,5)P₃ appears to be the best substrate for TP1P, other 3-phosphorylated phosphoinositides cannot be excluded as physiological substrates. Since it is not clear which 3-phosphorylated phosphoinositide species are present in the ER, and what their functions might be [26], resolution of this issue will require significant further investigation.

Our results indicate that TP1P α , TP1P β or TPTE β do not regulate the phosphorylation of PKB. Since PKB is believed to be activated by plasma membrane PtdIns(3,4,5)P₃ [and possibly PtdIns(3,4)P₂], and TP1P α , TP1P β and TPTE are localized on the ER or lack phosphatase activity, this would be predicted. It will be extremely interesting to see whether an active form of TP1P that is not restricted to intracellular membranes is expressed in any tissues, as this might be expected to regulate total cellular PtdIns(3,4,5)P₃ and PKB, and to be a potential tumour suppressor.

We thank Ian Batty and George McConnachie (Inositol Lipid Signalling Laboratory, University of Dundee) for assistance with the lipid phosphatase assays, John Lucocq (School of Life Sciences, University of Dundee) for advice and reagents for the colocalization studies, and Alex Gray (Division of Signal Transduction Therapy, University of Dundee) for discussions and advice. This work was supported by a studentship from the Medical Research Council (U.K.) to S.M.W. Work in the laboratory of C.P.D. is supported by the Medical Research Council, Zeneca, Boehringer Ingelheim, GlaxoSmithKline, Novo-Nordisk and Pfizer.

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Received 12 September 2001/27 September 2001; accepted 16 October 2001