Interaction of the protein tyrosine phosphatase PTPL1 with the PtdIns(3,4)P_2-binding adaptor protein TAPP1

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It has been postulated that PtdIns(3,4)P_2, one of the immediate breakdown products of PtdIns(3,4,5)P_3, functions as a signalling molecule in insulin- and growth-factor-stimulated pathways. To date, the **t**andem-**P**H-domain-containing **p**rotein-**1** (TAPP1) and related TAPP2 are still the only known PH-domain-containing proteins that interact strongly and specifically with PtdIns(3,4)P_2. In this study we demonstrate that endogenously expressed TAPP1, is constitutively associated with the protein-tyrosine-phosphatase-like protein-1 (PTPL1 also known as FAP-1). We show that PTPL1 binds to TAPP1 and TAPP2, principally though its first PDZ domain [where PDZ is postsynaptic density protein (**P**SD-95)/*Drosophila* disc large tumour suppressor (**d**lg)/tight junction protein (**Z**O1)] and show that this renders PTPL1 capable of associating with PtdIns(3,4)P_2 *in vitro*. Our data suggest that the binding of TAPP1 to PTPL1 does not influence PTPL1 phosphatase activity, but instead functions to maintain PTPL1 in the cytoplasm. Following stimulation of cells with hydrogen peroxide to induce PtdIns(3,4)P_2 production, PTPL1, complexed to TAPP1, translocates to the plasma membrane. This study provides the first evidence that TAPP1 and PtdIns(3,4)P_2 could function to regulate the membrane localization of PTPL1. We speculate that if PTPL1 was recruited to the plasma membrane by increasing levels of PtdIns(3,4)P_2, it could trigger a negative feedback loop in which phosphoinositide-3-kinase-dependent or other signalling pathways could be switched off by the phosphatase-catalysed dephosphorylation of receptor tyrosine kinases or tyrosine phosphorylated adaptor proteins such as IRS1 or IRS2. Consistent with this notion we observed RNA-interference-mediated knock-down of TAPP1 in HEK-293 cells, enhanced activation and phosphorylation of PKB following IGF1 stimulation.

Key words: adapter protein, phosphoinositide (PI), PI 3-kinase, protein dephosphorylation, scaffolding protein.

INTRODUCTION

Insulin and growth factors mediate many of their cellular effects through activation of phosphoinositide 3-kinase (PI 3-kinase), which phosphorylates PtdIns(4,5)P_2 to generate the second messenger PtdIns(3,4,5)P_3 [1]. PtdIns(3,4,5)P_3 triggers the activation of downstream signalling pathways by recruiting signalling proteins containing pleckstrin homology (PH) domains to the plasma membrane [2].

PtdIns(3,4,5)P_3 is metabolized in two ways. It can either be dephosphorylated at the 3 position by the PTEN (phosphatase and tensin homologue deleted on chromosome 10) phosphatase to generate PtdIns(4,5)P_2 or at the 5 position by SHIP1 and SHIP2 (Src homology 2 domain-containing inositol 5-phosphatases) to generate PtdIns(3,4)P_2 [3]. It has been suggested that PtdIns(3,4)P_2 may function as a signalling lipid, as its cellular concentrations are low in unstimulated cells and markedly elevated after activation of PI 3-kinase [1,4,5]. Moreover in certain cellular systems, such as an integrin-signalling pathway in platelets, PtdIns(3,4)P_2 can be generated independently from PtdIns(3,4,5)P_3 by phosphorylation of PtdIns3*P* catalysed by a phosphoinositide 4-kinase [6].

Several PH-domain-containing proteins such as PKB (protein kinase B) [7], PDK1 (3-phosphoinositide-dependent protein kinase-1) [7–9] and DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositides) [10], bind PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with similar affinity. Structural studies of the PH domains of PKB [11] and DAPP1 [12], demonstrate that basic residues on these proteins form specific interactions with the 3 and 4 inositol phosphate groups but the 5 phosphate is solvent-exposed and does not bind residues on the PH domain. To date only the tandem-PH-domain-containing protein (TAPP)-1, TAPP2 [13] and the PX (phox homology) domain of the phagocytic p47(phox) cytosolic subunit required for activation of NADPH oxidase, possess the properties of binding PtdIns(3,4)P_2 specifically [14,15]. However, it should be noted that TAPP1 possesses over 1000-fold higher affinity for PtdIns(3,4)P_2 than does the PX domain of p47(phox) [15].

TAPP1 and TAPP2 are putative adaptor proteins consisting of two sequential PH domains in which the C-terminal PH domain binds PtdIns(3,4)P_2. The N-terminal PH domain does not interact with any lipid tested [13]. The structure of the C-terminal PH domain of TAPP1, coupled with mutagenesis studies, suggested that basic residues interacted with the 3 and the 4 phosphate groups

Abbreviations used: DAPP1, dual adaptor for phosphotyrosine and 3-phosphoinositides; ECL®, enhanced chemiluminescene; FERM, four-point-one, ezrin, radixin, moesin; FSG, fish skin gelatin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HA, haemagglutinin; IGF, insulin-like growth factor; MUPP1, multi-PDZ-domain protein-1; PDK1, 3-phosphoinositide-dependent protein kinase-1; PDZ, postsynaptic density protein (**P**SD-95)/*Drosophila* disc large tumour suppressor (**d**lg)/tight junction protein (**Z**O1); PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PNPP, *p*-nitrophenyl phosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PTPL1 (also known as FAP-1 or Fas-associated phosphatase-1), protein-tyrosine-phosphatase-like protein-1; PTP-MEG1, megakaryocyte protein-tyrosine-phosphatase; PX, phox homology; SHIP, Src homology 2 domain-containing inositol 5-phosphatase; siRNA, small interfering RNA; TAPP, **t**andem-**P**H-domain-containing **p**rotein; TAPP1ΔCT, TAPP1 lacking the C-terminal 4 amino acids; TAPP1[R28L] etc., TAPP1 in which Arg-28 has been mutated to Leu etc.; YFP, yellow fluorescent protein.

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of PtdIns(3,4)P_2. Binding of TAPP1 to PtdIns(3,4,5)P_3 is likely to be sterically hindered by an alanine residue being located close to the position in which the 5 phosphate would be expected to reside [16]. Consistent with the notion that TAPP1 interacts with PtdIns(3,4)P_2 *in vivo*, we demonstrated that TAPP1 relocated from the cytosol to the plasma membrane of cells, following stimulation with agonists that induced PtdIns(3,4)P_2, but not with those that mainly induced PtdIns(3,4,5)P_3 [17]. Similarly, in B cells, both TAPP1 and TAPP2 were shown to translocate to the plasma membrane in response to antigen stimulation and this correlated with the formation of PtdIns(3,4)P_2 rather than production of PtdIns(3,4,5)P_3 [18]. Similar results have also been obtained in T-cell lines [19]. Although we have been unable to detect significant interaction of TAPP1 with PtdIns(3,4,5)P_3, another group has provided evidence that *in vitro* TAPP1 may interact with PtdIns(3,4,5)P_3, albeit more weakly than with PtdIns(3,4)P_2 [12].

Stimulation of cells with insulin and growth factors frequently results in a transient production of PtdIns(3,4,5)P_3, the level of which peaks after a few minutes and declines thereafter [1,5]. The mechanism by which PtdIns(3,4,5)P_3 declines after prolonged insulin and growth factor stimulation is poorly defined. One possibility is that PtdIns(3,4)P_2, which is generated after PtdIns(3,4,5)P_3 production, could serve as a specific negative feedback signal, recruiting to the plasma membrane PtdIns(3,4,5)P_3 phosphatases and/or protein tyrosine phosphatases. The latter could dephosphorylate receptors and adaptor subunits leading to inactivation of PI 3-kinase and a reduction in the level of PtdIns(3,4,5)P_3. In this study we provide evidence that might support this hypothesis by identifying protein-tyrosine-phosphatase-like protein-1 (PTPL1) as an enzyme that binds specifically to TAPP1 and TAPP2.

EXPERIMENTAL

Materials

Protease-inhibitor-cocktail tablets were from Roche (Lewes, East Sussex, U.K.), foetal bovine serum and other tissue culture reagents were from Bio Whittaker (Wokingham, Berks., U.K.), *p*-nitrophenyl phosphate (PNPP) was from Sigma, glutathione–Sepharose was from Amersham Biosciences (Little Chalfont, Bucks., U.K.) and BIOMOL Green reagent was from BIOMOL research laboratories (Plymouth Meeting, PA, U.S.A.). The precast 4–12 %-(w/v)-polyacrylamide/SDS/Bis-Tris gradient gels, the 3–8 %-(w/v)-Tris-acetate gels and the 10 % Bis-Tris gels were from Invitrogen (Groningen, The Netherlands). GST (glutathione S-transferase)–TAPP1 was expressed in *Escherichia coli* as described previously [13]. Synthetic RNA duplexes were synthesized by Dharmacon (Lafayette, CO, U.S.A.). Lipofectamine 2000 and IGF-1 (insulin-like growth factor) were from Invitrogen, phosphoinositide derivatives were from Cell Signaling Technology (Beverley, MA, U.S.A.), Hybond-C extra was from Amersham Biosciences. The phosphorylated insulin receptor peptide was synthesized by Dr Graham Bloomberg (Bristol, U.K.). The GST–PH domain of phospholipase Cδ was described previously [13] and was a kind gift of Dr A. Gray.

Antibodies

Antibodies raised against the whole TAPP1 protein were described previously [17], antibodies raised against the first 300 amino acids of human PTPL1 (antibody 1) or the 20 C-terminal residues of human PTPL1 (antibody 2) were both purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Pre-immune IgG antibody was purified from non-immunized sheep serum using a protein G–Sepharose column. Secondary antibodies coupled to horseradish peroxidase, used for immunoblotting, were from Pierce (Tattenhall, Cheshire, U.K.), monoclonal antibodies recognizing GST-, HA (haemagglutinin)- and FLAG-epitope-tags were from Sigma. Phospho-PKB T308 and S473 antibodies were from Cell Signaling Technology, PKB-total antibody was raised against whole protein. For immunoprecipitation of PKB for protein kinase assays anti-PKB antibody was used that was raised against the residues 1–149 of human PKB (Upstate Biotechnology, Lake Placid, NY, U.S.A). Protein-G coupled to horseradish peroxidase, used when immunoblotting of immunoprecipitates was studied, was from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.). Alexa-Fluor-conjugated anti-mouse secondary antibody was from Molecular Probes (Europe BV, Leiden, The Netherlands). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was from Research Diagnostics, Flanders, NJ, U.S.A.

General methods and buffers

Restriction-enzyme digests, DNA ligations, site-directed mutagenesis and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing (performed by The Sequencing Service, School of Life Sciences, University of Dundee, Dundee, Scotland, U.K.), using Big Dye terminator chemistry (Applied Biosystems Inc., Warrington, Cheshire, U.K.) on Applied Biosystems automated DNA sequencers. Buffer A contained 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.27 M sucrose and 0.1 % (v/v) 2-mercaptoethanol. Buffer B contained 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA and 0.1 % (v/v) 2-mercaptoethanol. Buffer C contained 50 mM Tris/HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 % (w/v) Triton X-100, 1 mM Na_3VO_4, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose and 0.1 % (v/v) 2-mercaptoethanol and 'complete' proteinase-inhibitor cocktail (one tablet/25 ml). Buffer D contained 40 mM Hepes pH 7.5, 1 mM EDTA, 1 % (w/v) Triton X-100, 0.27 M sucrose, 0.1 % (v/v) 2-mercaptoethanol and 'complete' proteinase-inhibitor cocktail (one tablet/25 ml). Buffer E contained 3 % (w/v) fatty-acid-free BSA in 50 mM Tris/HCl pH 7.5, 150 mM NaCl and 0.1 % (v/v) Tween 20. Sample Buffer contained 50 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.005 % (w/v) Bromophenol Blue and 1 % (v/v) 2-mercaptoethanol. PtdIns(3,4,5)P_3 phosphatase assays were performed using PtdIns(3,4,5)P_3 labelled at the 3 position with [^{33}P]P_i, as described previously [20].

DNA constructs

Mammalian expression vectors encoding for N-terminal FLAG-epitope-tagged TAPP1, TAPP1ΔCT (lacks C-terminal 4 amino acids), TAPP1[R28L], TAPP1[R211L], TAPP2 and TAPP2ΔCT (lacks C-terminal 4 amino acids) and that encoding for the thirteenth PDZ domain of MUPP1 (multi-PDZ-domain protein-1; residues 1885–2042) in the pCMV5 vector or pEBG-2T vector (encodes a N-terminal GST tag) or pEYFP-C1 (ClonTech UK Limited, Basingstoke, U.K.), have been described previously [13,17]. Individual HA-tagged PTPL1-PDZ domains were amplified by PCR using a full-length human PTPL1 cDNA clone kindly provided by Dr C. H. Heldin (Ludwig Institute for Cancer Research, Biomedical Centre, Uppsala, Sweden) [21]. Information on primers used can be obtained by request. The PDZ domains 1–5 encompass residues 1099–1199, 1372–1467, 1506–1615, 1796–1887, and 1888–1990, respectively. The PCR

products were ligated into pCR2.1 TOPO vector (Invitrogen), sequenced and subcloned into the *Bam*H1 site of pEBG2T [22] and pCMV5 vectors [23] as a *Bam*H1–*Bam*H1 fragment (PDZ domains 1, 2 and 3) or as a *Bgl*II–*Bgl*II fragment (PDZ domains 4 and 5). Full-length PTPL1 with an N-terminal HA tag was generated by a combination of PCR and subcloning of the PTPL1 cDNA and subcloned into the pCMV5 and pEBG2T vectors. The phosphatase domain of PTPL1 (residues 2087–2485) was also amplified and subcloned into the pEBG2T vector. The HA-tagged PDZ domain of PTP-MEG1 (megakaryocyte protein-tyrosine-phosphatase; NCBI accession number P29074) covering amino acids 509–610 was amplified by PCR from an expressed sequence tag (NCBI accession number BI916937, IMAGE clone 5241923) and subcloned into the pEBG2T vector. The HA-tagged PDZ domain of PTPH1 (NCBI accession number NP_002820) covering residues 519–601 was amplified by PCR from an expressed sequence tag (NCBI accession number BG115423, IMAGE clone 4416683) and subcloned into the pEBG2T vector.

Cell culture and cell lysis

HEK-293 cells were cultured on 10-cm-diameter dishes in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum. Cells were lysed in ice-cold Buffer C, unless otherwise indicated, and the lysates centrifuged for 10 min at 16 000 ***g*** at 4 °C. Protein concentrations were determined using the Bradford method [23a] and BSA was employed as the standard.

Transient transfections of HEK-293 cells

HEK-293 cells cultured on 10-cm-diameter dishes were transfected with 5 μg of the indicated plasmid, using the modified calcium phosphate method [24]. At 36 h post-transfection the cells were lysed in 1 ml of Buffer C, unless otherwise indicated, and the lysates clarified by centrifugation at 13 000 ***g*** for 15 mins at 4 °C. For immunolocalization studies, HEK-293 cells were transfected with FuGene 6 transfection reagent (Roche) following manufacturer's protocol.

Purification of GST-fusion proteins from HEK-293 cell lysates

For the experiments performed in Figures 1, 3, 7 and 8, the clarified cell lysates derived from 20 dishes (10-cm-diameter) of HEK-293 cells lysed in Buffer D, were pooled and incubated with 0.25 ml of GST–Sepharose for 1 h at 4 °C. The beads were washed twice in Buffer D, followed by two further washes in Buffer A. The GST-fusion proteins were eluted by incubating the resin with an equal volume of Buffer A containing 40 mM glutathione and the eluted protein was collected after centrifugation through a Spin X column (Costar, High Wycombe, Bucks., U.K.). For the experiments performed in Figures 2 and 5, cells were lysed in Buffer C and following affinity purification glutathione–Sepharose beads washed in Buffer C containing 0.5 M NaCl followed by Buffer B.

Protein tyrosine phosphatase assay

Phosphatase activity was measured by monitoring hydrolysis of PNPP using a spectrophotometric assay which detects formation of the reaction product *p*-nitrophenol by absorbance at 405 nm [25] or monitoring the dephosphorylation of the insulin-receptor-peptide substrate (TRDIYETDYYRK), phosphorylated at the underlined tyrosine residues equivalent to Tyr-1158, Tyr-1162 and Tyr-1163 on the insulin receptor. For the PNPP assay immunoprecipitates or purified GST-fusion proteins (between 5–50 μg) were diluted to 0.1 ml in 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM DTT, 10 % (v/v) glycerol, pre-incubated at 30 °C with gentle agitation for 5 min before addition of 0.1 ml of solution containing 0.1 M imidazole pH 7.5, 40 mM PNPP and 20 mM DTT. Absorbance was measured at 405 nm either continually or at an end-point for periods of up to 120 min, using a Versa max absorbance plate reader with Softmax Pro 4.0 software (Molecular Devices, Wokingham, Berks., U.K.). Rates or amounts of *p*-nitrophenol production were calculated using a standard curve generated from known amounts of *p*-nitrophenol. For the insulin-receptor-peptide assay a 50 μl reaction was set up containing 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM DTT, 20 μM of the tyrosine-phosphorylated peptide and 100 ng of GST-PTPL1. After 10 min at 22 °C, the reactions were terminated by the addition of 0.1 ml of BIOMOL green reagent and after an additional 20 min the amount of phosphate liberated was quantified by absorbance at 620 nm and measured against P_i standards. In all assays we ensured that the rate of *p*-nitrophenol formation or the dephosphorylation of the insulin receptor peptide, was linear with time.

Immunoprecipitation of endogenous TAPP1 and PTPL1

The polyclonal anti-TAPP1 or the pre-immune IgG antibodies were conjugated to Protein G–Sepharose at a ratio of 1 μg of antibody to 1 μl of beads, and the commercial PTPL1 antibody 1 at a ratio of 0.5 μg of antibody to 1 μl of beads. These were incubated with 2.5 mg of clarified HEK-293 cell lysate in which the cells had been lysed in Buffer D. After 1 h at 4 °C with gentle agitation, the immunoprecipitates were washed twice with Buffer D containing 0.5 M NaCl, washed twice with Buffer B and either subjected to protein tyrosine phosphatase assay and/or to immunoblot analysis.

Immunoblotting

For blots of total cell lysates 20 μg of protein was used. For the purified GST-fusion proteins to detect association of endogenous PTPL1, 10 μg of fusion protein was employed for the PTPL1 blots and 0.1 μg for the GST blots. Samples were subjected to SDS/PAGE and transferred to nitrocellulose. The membranes were blocked in 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 0.5 % (v/v) Tween (TBS-Tween) and 10 % (w/v) skimmed milk for 2 h, then incubated in the same buffer for 7 h at 4 °C in the presence of 1 μg/ml of the indicated primary antibody. Detection was performed using the enhanced chemiluminescene (ECL®; Amersham Biosciences) reagent and horseradish peroxidase-conjugated secondary antibodies except for when co-immunoprecipitations were performed, where protein G conjugated to horseradish peroxidase was used.

Immunofluorescence

HEK-293 cells were cultured on poly-L-lysine-coated cover slips to 50 % confluence in 6-well plates and transfected with 1 μg of a construct encoding wild-type or mutant forms of YFP (yellow fluorescent protein)-TAPP1 in the presence or absence of 1 μg of HA-PTPL1 using FuGene 6 transfection reagent (Roche). Post transfection (24 h), cells were serum-starved for 16 h. Cells were either left unstimulated or treated with 1 mM H_2O_2 for 10 min. Cells were fixed in 4 % (w/v) paraformaldehyde in PHEM Buffer

(60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM $MgSO_4$, pH 7.0). The cells were washed twice with PBS and permeablized with 0.2 % Triton X-100 in PBS for 5 min, washed three times with 0.1 % (v/v) PBS-Tween before being blocked with 3 % fish skin gelatin (FSG) in PBS-Tween for 30 min. Cells were then incubated with anti-HA antibody at 2 μg/ml in 3 % FSG/PBS-Tween for 1 h, washed three times with PBS-Tween and incubated with anti-mouse antibody secondary labelled with Alexa Fluor 594 (Molecular Probes) in 3 % FSG/PBS-Tween for 30 min. The cells were washed three times with PBS-Tween and mounted on slides with Mowiol (Calbiochem, La Jolla, CA, U.S.A.). The cells were imaged and confocal sections collected, using a Zeiss LSM 510 META confocal microscope. Each channel was scanned independently to avoid cross talk (Multi-Tracking).

Protein–lipid-overlay assay

In order to assess lipid binding we employed the protein–lipid-overlay assay that we have described previously [26]. Briefly, 1 μl of lipid solution containing 1–250 pmol of phospholipids dissolved in a mixture of chloroform/methanol/water (1:2:0.8, by vol.) was spotted on to Hybond-C extra membrane and allowed to dry at 22 °C for 1 h. The membranes were blocked in Buffer E for 1 h. GST–HA-PTPL1 (1 nM), expressed and purified from HEK-293 cells as described above, was preincubated on ice in the presence or absence of wild-type and mutant forms of 10 nM GST–Flag-TAPP1 in Buffer E, also derived from a HEK-293 cell expression as described previously [17]. The mixture was then incubated overnight with the Hybond-C membranes at 4 °C, with gentle agitation. The membranes were washed six times with Buffer E not containing BSA and then incubated with 1 μg/ml anti-HA monoclonal antibody for 2 h at 22 °C. The membranes were washed again as before, then incubated for 2 h with a 1:5000 dilution of anti-mouse HRP conjugate. Finally the membranes were washed 12 times over 1 h and the HA-PTPL1 that had bound to the membrane through the interaction of the lipid or through the binding of TAPP1 was detected by ECL®.

Small-interfering-RNA (siRNA) studies

Several RNA duplexes were raised against the human TAPP1 sequence and were selected by the siDESIGN centre programme at http://www.dharmacon.com/. The most effective at reducing endogenous TAPP1 levels possessed the sequence (5′-C CAC CUC AAG AUA GUG CGG-3′). As a control a RNA duplex that does not target any cellular mRNA (5′-G UGC GCA CGU AGA UAG AGU-3′) was employed. HEK-293 cells were cultured in 6-well plates to 30 % confluence and transfected with 100 pmol of TAPP1 or control RNA duplex per well using Lipofectamine 2000, following the manufacturer's protocol. Post transfection (24 h) the cell medium was replaced with fresh Dulbecco's modified Eagle's medium containing 10 % fetal calf serum. The cells were left for 32 h, then deprived of serum for a further 16 h. The cells were either left unstimulated or stimulated with 50 ng/ml IGF1 for 5 min prior to lysis. PKB activity was determined following its precipitation and assay using the Crosstide peptide as a substrate (GRPRTSSFAEG), as described previously [27].

RESULTS

Evidence that TAPP1 interacts with a protein tyrosine phosphatase

In order to test the hypothesis that TAPP1 and/or TAPP2 interacted with a PtdIns(3,4,5)P_3 phosphatase and/or a protein tyrosine phosphatase (see Introduction), GST–TAPP1 or GST–TAPP2, expressed in HEK-293 cells, were affinity purified on glutathione–Sepharose and incubated with either ^{33}P-labelled PtdIns(3,4,5)P_3 (labelled at the 3 position) or PNPP to measure tyrosine phosphatase activity. Although no PtdIns(3,4,5)P_3 phosphatase activity was observed (results not shown), significant PNPP phosphatase activity was associated with TAPP1 and to a lesser extent with TAPP2 (Figure 1A).

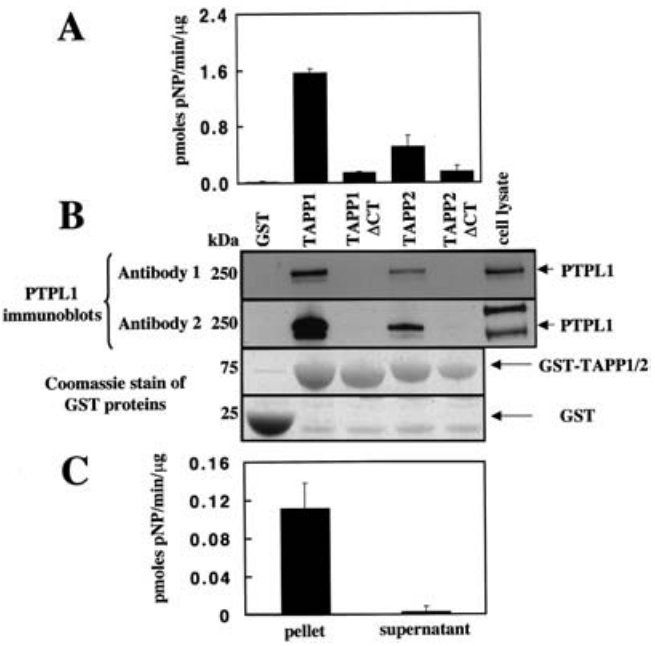

Figure 1 Binding of TAPP1 and TAPP2 to PTPL1

(**A**) HEK-293 cells were transiently transfected with DNA constructs encoding for the indicated GST-fusion proteins of the wild-type and mutant forms of TAPP1 and TAPP2. Post-transfection (36 h) cells were lysed and the GST-fusion proteins purified by affinity chromatography on glutathione–Sepharose beads. Equal amounts of purified protein were assayed for PNPP phosphatase activity. The results are presented as pmoles of pNP produced/min/μg of TAPP protein added to the assay. Results are the mean ± S.E.M. of two separate experiments in which each condition was assayed in duplicate. (**B**) The indicated purified GST-fusion proteins were electrophoresed on a 3–8 % (w/v) polyacrylamide gel and immunoblotted with two separate anti-PTPL1 antibodies. The GST proteins were also electrophoresed on a 4–12 % (w/v) polyacrylamide gel, which was stained with Coomassie Brilliant Blue to detect expression of GST as well as wild-type and mutant GST–TAPP1 and GST–TAPP2. The position of the molecular-mass markers (Bio-Rad precision markers) is indicated (kDa). Similar results were obtained in three separate experiments. (**C**) Equal amounts of GST–TAPP1 protein that had been purified from HEK-293 cell lysates as described in (**A**), were incubated with the 'PTPL1 antibody 1' conjugated to Protein-G–Sepharose, and following incubation, the PNPP phosphatase activity in the supernatant and washed-pellet (pellet) fraction of the immunoprecipitation was measured. The results shown are the mean ± S.D. for a single experiment in which each determination was carried out in triplicate. Similar results were obtained in three separate experiments.

Evidence that TAPP1/TAPP2 specifically associate with PTPL1

We found previously that the four C-terminal amino acids of TAPP1 and TAPP2 comprise a PDZ binding motif that may enable them to interact with PDZ-domain-containing protein(s) [17]. We found that TAPP1ΔCT and TAPP2ΔCT mutants, lacking the four C-terminal residues, were associated with far lower levels of PNPP phosphatase compared with the wild-type proteins (Figure 1A), indicating that the TAPP-interacting tyrosine phosphatase might contain a PDZ domain. Analysis of protein sequence databases, revealed that only three protein tyrosine phosphatases

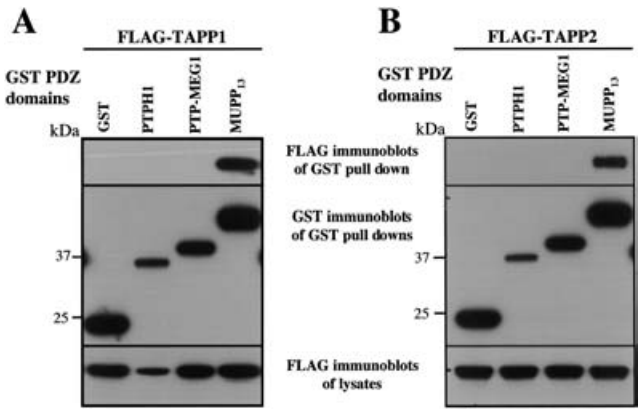

Figure 2 TAPP1 does not bind the PDZ domains of PTP-MEG1 or PTPH1

HEK-293 cells were co-transfected with plasmids encoding for FLAG-TAPP1 (**A**) or FLAG-TAPP2 (**B**) in the presence of constructs encoding for the expression of GST, GST–PTPH1-PDZ domain (PTPH1), GST–PTP-MEG1-PDZ domain (PTP-MEG1) or PDZ domain 13 of MUPP1 ($MUPP_{13}$). Post transfection (36 h), the GST-fusion proteins were purified by glutathione–Sepharose and immunoblotted using the anti-FLAG antibody to detect the presence of TAPP1/TAPP2 proteins or with an anti-GST antibody to visualize GST-fusion proteins. Similar results were obtained in two separate experiments, which were each analysed in duplicate. The position of the molecular-mass markers is indicated (kDa).

contain PDZ domains, namely PTPL1 [21,28], PTPH1 [29] and PTP-MEG1 [30]. To test whether PTPL1 was associated with GST–TAPP1 and GST–TAPP2, the GST–TAPP proteins purified from cell extracts on glutathione–Sepharose, were immunoblotted with two commercially available anti-PTPL1 antibodies. We observed that both antibodies recognized a protein of the correct molecular mass for PTPL1 (~260 kDa), which was associated with GST–TAPP1 and GST–TAPP2, but not the GST–TAPP1ΔCT and GST–TAPP2ΔCT mutants (Figure 1B). As we were unable to obtain antibodies that recognised PTPH1 or PTP-MEG1 in HEK-293 cell lysates, we cloned the single PDZ domain of these tyrosine phosphatases and tested whether they could bind TAPP1 and TAPP2 in a co-transfection binding assay in HEK-293 cells. In Figure 2, we demonstrate that TAPP1 and TAPP2 failed to interact with either the PDZ domain of PTPH1 or PTP-MEG1, whilst in parallel experiments they bound to a PDZ domain of MUPP1, previously shown to bind TAPP1 and TAPP2 [17].

We investigated next whether the tyrosine phosphatase activity associated with TAPP1 could be immunoprecipitated with an anti-PTPL1 antibody. In Figure 1(C), we demonstrate that all detectable tyrosine phosphatase activity associated with GST–TAPP1 was immunoprecipitated with a PTPL1 antibody, suggesting that the bulk of the PNPP-tyrosine-phosphatase activity associated with TAPP1 can be attributed to PTPL1.

Binding of PTPL1 to TAPP1 and TAPP2 was specific, as no PTPL1 protein or PNPP phosphatase activity was associated with either GST alone or GST-fusion proteins of two other PH domains, DAPP1 and FAPP1, or SGK (serum- and glucocorticoid-induced protein kinase-1), expressed in HEK-293 cells (Figure 3A). Moreover, consistent with the notion that TAPP1 was interacting with PTPL1 through its C-terminal PDZ-domain-binding motif, we found that GST–TAPP1 mutants in which either the N-terminal PH domain (TAPP1[R28L]) or the C-terminal PH domain (TAPP1[R211L]) were disrupted [13], were still able to interact with endogenously expressed PTPL1 to the same extent as wild-type TAPP1 in HEK-293 cells, judged by tyrosine phosphatase activity and immunoblot analysis (Figure 3B).

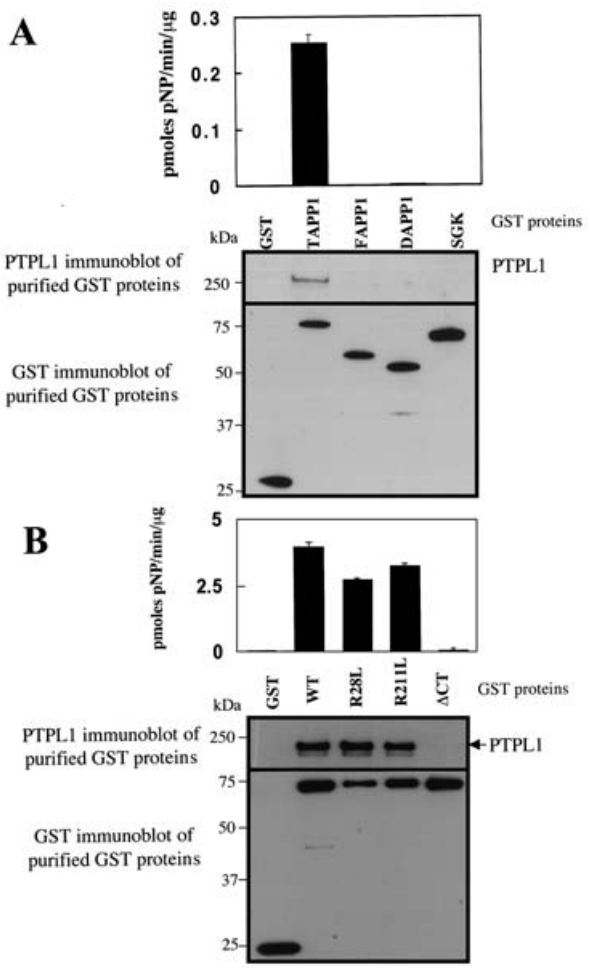

Figure 3 PTPL1 binds TAPP1 specifically

(**A**) HEK-293 cells were transiently transfected with plasmids encoding DNA for the expression of GST, GST–TAPP1, GST–FAPP1, GST–DAPP1 and GST–SGK. Post transfection (36 h), GST-fusion proteins were purified using glutathione–Sepharose and assayed for PNPP phosphatase activity and immunoblotted with an anti-PTPL1 antibody (antibody 1) and an anti-GST antibody. The results are presented as pmoles of pNP produced/min/μg of GST-fusion protein added to the assay. PNPP-phosphatase-activity assays were carried out in triplicate and results are presented as the mean $\pm$ S.D. for a single experiment. Similar results were obtained in two separate experiments. (**B**) As above except that HEK-293 cells were transfected with GST and the wild-type (WT) or indicated mutants of GST–TAPP1 (R28L, R211L, ΔCT), respectively. The position of the molecular-mass markers is indicated (kDa).

Endogenous TAPP1 interacts with endogenous PTPL1

Next we immunoprecipitated endogenous TAPP1 from HEK-293 cells and assessed whether it was associated with PNPP phosphatase activity and PTPL1 protein. As a control, immunoprecipitations were performed from the same amount of lysates with pre-immune IgG and anti-PTPL1 antibodies. The endogenous TAPP1 immunoprecipitates were associated with significant levels of PNPP phosphatase, which was absent from the immunoprecipitates obtained using pre-immune IgG (Figure 4A). Approx. 6-fold higher levels of PNPP phosphatase activity were immunoprecipitated with the anti-PTPL1 antibody compared with the TAPP1 antibody, suggesting that not all PTPL1 is associated with TAPP1, or that the binding of TAPP1 to PTPL1 suppressed its activity. Immunoblotting studies also confirmed that PTPL1 was efficiently co-immunoprecipitated with the anti-TAPP1

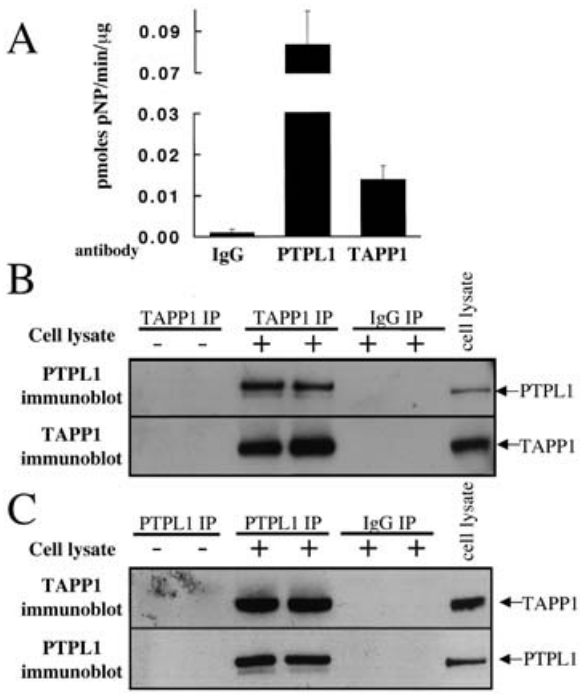

Figure 4 Endogenous TAPP1 is associated with PTPL1

(**A**) TAPP1 or PTPL1 were immunoprecipitated from 2 mg of HEK-293 cell lysate using 10 μg of anti-TAPP1 antibody (TAPP1), 6 μg of anti-PTPL1 antibody (PTPL1 or antibody 1), or 10 μg of pre-immune IgG (IgG), covalently coupled to protein-G–Sepharose. The immunoprecipitates were assayed for PNPP phosphatase activity (**A**) or immunoblotted with the indicated antibodies (**B** and **C**). The results of the PNPP phosphatase activity are presented as pmoles of pNP produced/min/μg of total protein immunoprecipitated from the cell lysate. In each gel, 20 μg of total cell lysate was also immunoblotted in parallel. PNPP phosphatase activity assays were carried out in triplicate and results are presented as the mean $\pm$ S.D. for a single experiment. Similar results were obtained in two separate experiments. The position of the molecular-mass markers is indicated (kDa).

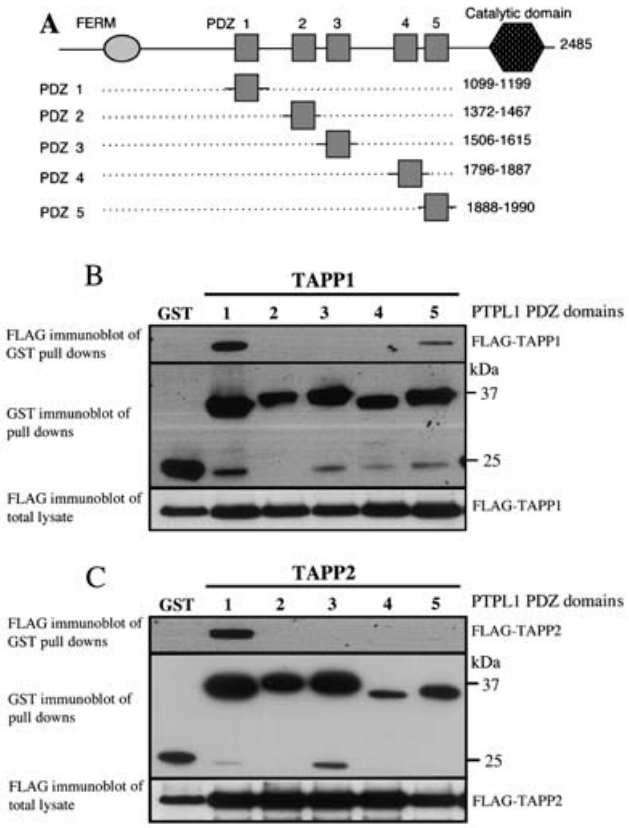

Figure 5 TAPP1 and TAPP2 bind to the first PDZ domain of PTPL1

(**A**) Schematic diagram of the domains in PTPL1 and the PDZ 1–5 domains expressed in HEK-293 cells. HEK-293 cells were co-transfected with DNA encoding FLAG-TAPP1 (**B**) or FLAG-TAPP2 (**C**) together with GST alone or the indicated PTPL1 GST-PDZ (1–5) domains. Post transfection (36 h), the GST-fusion proteins were affinity purified on glutathione–Sepharose and analysed by immunoblotting with anti-FLAG antibody to detect FLAG-TAPP1 or FLAG-TAPP2 or with an anti-GST antibody to visualize the levels of the GST-fusion proteins. Prior to affinity purification cell lysates were also immunoblotted with anti-FLAG antibody to ensure levels of TAPP1 and TAPP2 were similar for each condition. Similar results were obtained in three separate experiments.

antibody, but not with a pre-immune IgG antibody (Figure 4B, upper panel). Consistent with PTPL1 and TAPP1 being in a complex, endogenous PTPL1 immunoprecipitated from HEK-293 cells, was shown to be associated with TAPP1 (Figure 4C, upper panel). In addition, we also stimulated HEK-293 cells with hydrogen peroxide to induce PtdIns(3,4)P_2 production, with only minor PtdIns(3,4,5)P_3 formation [31], but this did not affect the amount of PTPL1 co-purified with TAPP1, indicating that these proteins are constitutively associated (results not shown).

TAPP1 and TAPP2 interact with the first PDZ domain of PTPL1

PTPL1 is a large protein comprising 2485 residues, in which the five PDZ domains are located between residues 1102 and 1990, while the phosphatase domain is located at the C-terminus between residues 2087 and 2485 (Figure 5A). As the TAPP1ΔCT and TAPP2ΔCT mutants failed to interact with PTPL1 (Figures 1A and 1B), this indicated that TAPP1 and TAPP2 interacted with one or more of the PDZ domains of PTPL1. Using a co-expression binding assay, we tested the ability of the five isolated PDZ domains of PTPL1 to interact with TAPP1 (Figure 5B) and TAPP2 (Figure 5C). We found that the first PDZ domain of PTPL1 bound to both TAPP1 and TAPP2, whereas in parallel experiments the PDZ domains 2, 3 and 4 of PTPL1, failed to bind. We consistently observed that PDZ domain 5 of PTPL1, interacted weakly with TAPP1, but not TAPP2 (Figure 5B), which might explain why TAPP1 appears to interact more strongly with PTPL1 than TAPP2 (Figures 1A and 1B).

TAPP1 influences the localization of PTPL1

HEK-293 cells were transfected with tagged versions of PTPL1 and/or TAPP1 to enable the localization of these proteins to be visualized independently in the same cell by confocal fluorescence microscopy. PTPL1, expressed on its own, was located at the plasma membrane and, to a lesser extent, in the cytoplasm (Figure 6A, panel 1), as reported previously [32–34]. The binding of PTPL1 to the plasma membrane has been shown recently to be mediated through a FERM (four-point-one, ezrin, radixin, moesin) domain located at the N-terminus of PTPL1 [35]. TAPP1, on its own, was localized predominantly in the cytosol of unstimulated cells (Figure 6A, panel 2), as reported previously [17,18]. When PTPL1 and TAPP1 were co-expressed, co-localization was observed in the cell cytoplasm and significantly lower levels of PTPL1 were found at the plasma membrane compared with PTPL1 expressed alone (Figure 6A, compare panels 1 and 3). Moreover, this effect of TAPP1 on PTPL1 localization was even more evident when PTPL1 was co-expressed with the

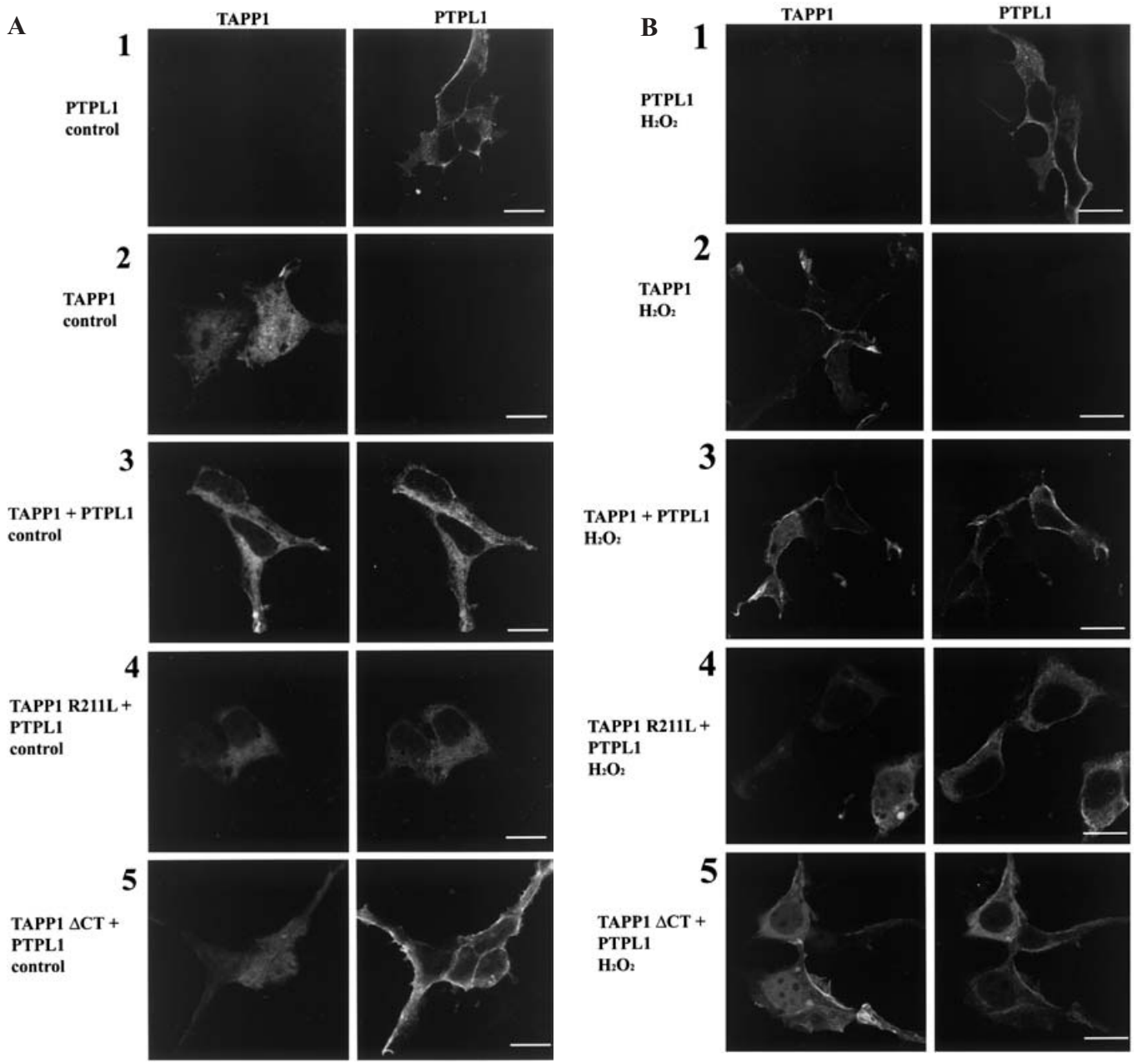

Figure 6 TAPP1 anchors PTPL1 in the cytoplasm away from the plasma membrane

(**A** and **B**) HEK-293 cells were transfected with the indicated constructs encoding for the expression of the wild-type or indicated mutants of YFP-TAPP1 (TAPP1) or HA-PTPL1 (PTPL1). Post transfection (24 h) the cells were deprived of serum for 16 h and either left unstimulated (**A**) or stimulated with 1 mM H_2O_2 for 10 min (**B**). The cells were fixed in 4 % (w/v) paraformaldehyde and immunostained with anti-HA antibody to detect PTPL1 (594 Alexa Fluor anti-mouse secondary antibody, red channel) and YFP-TAPP1 localization was visualized directly through the YFP fluorescence (green channel). Confocal image sections were taken using a Zeiss LSM 510 META confocal microscope. The cells shown are representative images obtained in 3 separate experiments. The scale bars correspond to 10 μm.

non-PtdIns(3,4)P_2 binding TAPP1[R211L] mutant [17] (Figure 6A, panel 4). The TAPP1ΔCT mutant, which does not bind PTPL1, failed to induce cytoplasmic localization of PTPL1 (Figure 6A, panel 5). TAPP1 translocates to the plasma membrane in response to stimuli such as H_2O_2 that induce the formation of PtdIns(3,4)P_2 (Figure 6B, panel 2), as reported previously [17]. The cellular localization of PTPL1 expressed alone is not altered by exposure to H_2O_2 (Figure 6B, panel 1), while TAPP1 was translocated to the plasma membrane whether or not it was co-expressed with PTPL1 (Figure 6B, panels 2 and 3). When PTPL1 was co-expressed with TAPP1 (Figure 6B, panel 3), but not mutant TAPP1[R211L] (Figure 6B, panel 4), significantly higher levels of PTPL1 were found at the plasma membrane of H_2O_2-stimulated cells compared with unstimulated cells.

Evidence that TAPP1 enables PTPL1 to associate with PtdIns(3,4)P_2

As discussed above, the FERM domain of PTPL1 enables it to associate with plasma membranes and it has been suggested

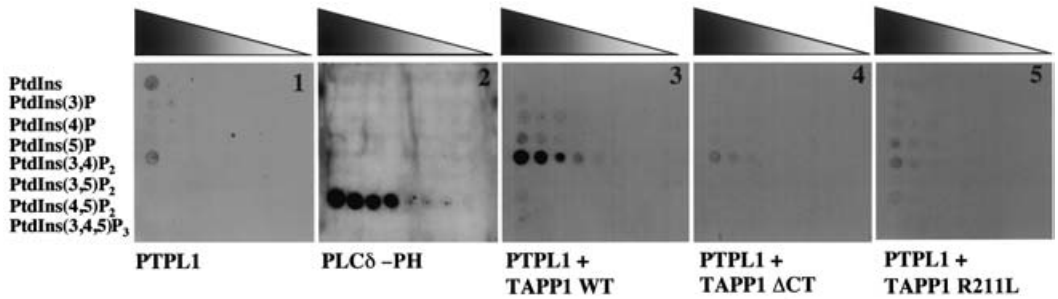

Figure 7 TAPP1 localizes PTPL1 to PtdIns(3,4)P_2

The ability of PTPL1 to bind phosphoinositides in the presence or absence of wild-type or indicated mutant forms of TAPP1 were analysed using a protein–lipid-overlay assay as described in the experimental procedures. Serial dilutions of the indicated phosphoinositides (250 pmol, 100 pmol, 50 pmol, 25 pmol, 12.5 pmol, 6.3 pmol, 3.1 pmol and 1.6 pmol, respectively) were spotted on to nitrocellulose membranes (left to right across each membrane), which were then incubated with purified GST–HA-PTPL1 either with or without the indicated forms of TAPP1 (TAPP1 WT, TAPP1 ΔCT, TAPP1 R211L). The PTPL1 that had bound to the membrane was detected using an HA antibody. As a control the PH domain of GST–phospholipase Cδ (PLCδ-PH) was used at identical concentrations to PTPL1 (1 nM) and binding was detected using an anti-GST antibody. Results shown are representative of three separate experiments.

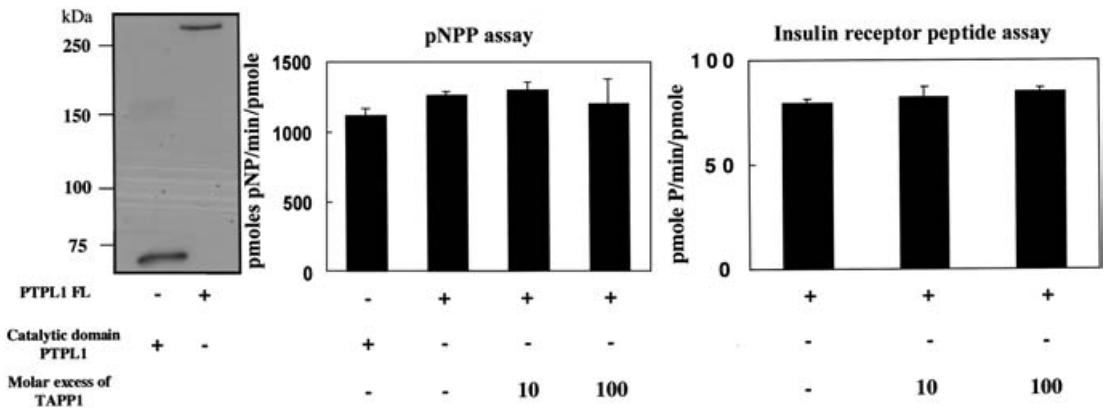

Figure 8 Activity of PTPL1 is not influenced by PDZ domains or binding of TAPP1

HEK-293 cells were transfected (+) with DNA encoding for GST-fusion proteins of full-length PTPL1 (PTPL1 FL) or a catalytic fragment of PTPL1 that lacks the PDZ domains (residues 2087–2485). Post transfection (36 h), these GST-fusion proteins were affinity purified on glutathione–Sepharose. Purified GST-fusion protein was immunoblotted with anti-GST antibody. Prior to assay equal molar amounts of purified phosphatase were incubated in the absence or presence of a 10-fold (10) or 100-fold (100) molar excess of bacterially expressed and purified GST–TAPP1 for 10 min at 22 °C. Assays were initiated by the addition of either PNPP or the phosphorylated insulin receptor peptide. The results of the PNPP phosphatase activity (PNPP assay) are presented as pmol of pNP produced/min/pmol of GST–PTPL1 fusion protein added to the assay, and for the insulin receptor peptide assay presented as pmol of phosphate released/min/pmol of GST–PTPL1 protein added to the assay. Results are presented as the mean ± S.D. of a single experiment with each condition assayed in triplicate. Similar results were obtained in two separate experiments.

that this could be mediated through the binding of the FERM domain to PtdIns(4,5)P_2 [35]. However, the evidence that the FERM domain interacts with PtdIns(4,5)P_2 specifically is weak. Consistent with this, employing a protein–lipid-overlay assay, we were unable to detect any significant interaction of HA-epitope-tagged purified PTPL1 with any phosphoinositide tested employing a HA antibody to specifically detect PTPL1 (Figure 7, panel 1). As a control we demonstrate that the PH domain of phospholipase Cδ, in a parallel experiment, bound strongly to PtdIns(4,5)P_2 (Figure 7, panel 2). Addition of a 10-fold excess of wild-type TAPP1 to PTPL1, resulted in association of PTPL1 with PtdIns(3,4)P_2 (Figure 7, panel 3). TAPP1 in the absence of PTPL1 was not detected bound to PtdIns(3,4)P_2 as it is not HA-tagged and does not cross react with the HA antibody (results not shown). As a further control we demonstrated that a mutant of TAPP1, lacking the C-terminal PDZ-binding motif that can not bind PTPL1 (Figure 1), does not localize PTPL1 to PtdIns(3,4)P_2 (Figure 7, panel 4). Moreover, the non-PtdIns(3,4)P_2-binding TAPP1[R211L] mutant that still interacts with PTPL1 (Figure 3B), also failed to localize PTPL1 to PtdIns(3,4)P_2 (Figure 7, panel 5). These results show that TAPP1 can recruit PTPL1 to PtdIns(3,4)P_2 *in vitro*.

TAPP1 does not influence the activity of PTPL1

We wished next to evaluate whether the PDZ domains of PTPL1, or binding of TAPP1 to PTPL1, influenced the PNPP phosphatase activity or the ability of PTPL1 to dephosphorylate an insulin-receptor-peptide substrate phosphorylated at residues equivalent to Tyr-1158, Tyr-1162 and Tyr-1163 of the human insulin receptor. In order to do this we expressed either full-length GST–PTPL1 or a catalytic fragment lacking all of the PDZ domains (residues 2087–2485) in HEK-293 cells. Interestingly, the specific PNPP phosphatase activity of the full-length PTPL1 was not significantly different to that of the isolated catalytic domain (Figure 8). Moreover, addition of up to 100-fold molar excess of TAPP1 to full-length PTPL1, did not alter phosphatase activity assessed with either substrate (Figure 8).

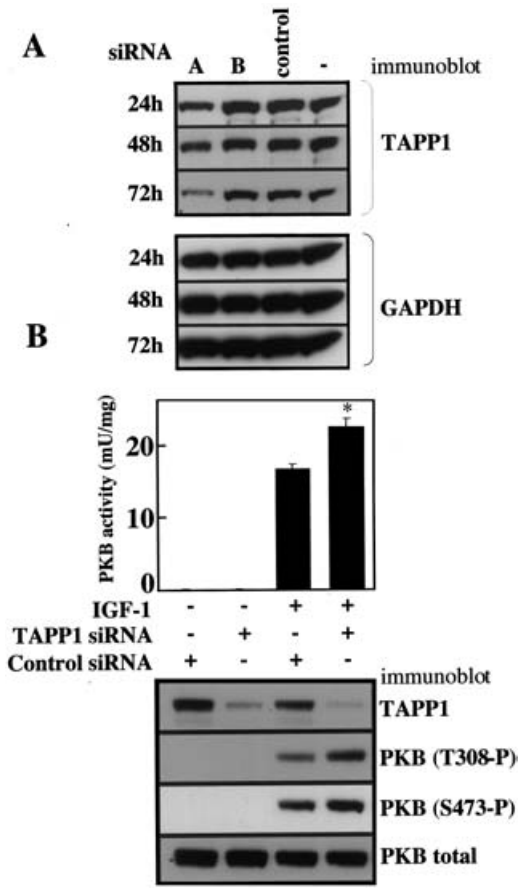

Figure 9 Knock-down of TAPP1 moderately enhances PKB activation in response to IGF1

(**A**) HEK-293 cells were either not transfected or transfected with the indicated TAPP1 RNAi duplexes or a control RNAi duplex. At the indicated times post-transfection, cells were lysed and 20 μg of lysate were immunoblotted with the TAPP1 or GAPDH antibodies. (**B**) as above except that 56 h post-transfection cells were deprived of serum for a further 16 h and left unstimulated (−) or stimulated (+) with 50 ng/ml IGF1 for 5 min. Cells were lysed and PKB immunoprecipitated and assayed using the Crosstide peptide as a substrate. The results are presented as mUnits activity/mg lysate (mU/mg) mean ± S.E.M. where 1 mU represents 1 pmol of substrate phosphorylated in 1 min. Levels of TAPP1, PKB protein and phosphorylation of PKB at Thr-308 and Ser-473 were analysed by immunoblotting with appropriate antibodies (T308 and S473). Stimulations were carried out in duplicate with each duplicate sample being assayed in triplicate. Results are representative of two separate experiments. *, $P < 0.005$ employing the Student's *t* test.

siRNA depletion of TAPP1 enhances PKB phosphorylation and activation

To examine the role of TAPP1 in regulating the PI 3-kinase pathway, we employed a specific siRNA approach, by taking advantage of the ability to transfect cells with small RNA duplexes to deplete mRNA and protein levels [36]. We evaluated several siRNA sequences for their ability to reduce TAPP1 protein levels over a 72 h period in HEK-293 cells. One of these sequences (duplex A, Figure 9A), reduced the level of endogenous TAPP1 by 80–90 % over a 72 h period, without affecting levels of GAPDH. In order to assess how knock-down of TAPP1 might affect a cellular response regulated by PI 3-kinase, we measured activation of PKB employing a quantitative immunoprecipitation kinase assay as well as analysing phosphorylation of its two major activating phosphorylation sites (Thr-308 and Ser-473). Knock-down of TAPP1 did not affect the basal PKB activity or phosphorylation in serum-starved cells. However, following 5 min of IGF1 stimulation and in comparison with cells transfected with a control RNAi duplex, in cells in which TAPP1 expression had been reduced, an increase in phosphorylation of PKBα at Thr-308 and Ser-473 was observed. This was accompanied by a moderate but statistically significant approx. 20–30 % enhancement of PKB activity.

DISCUSSION

PTPL1 is a widely expressed [21,37] large protein tyrosine phosphatase consisting of a FERM domain at the N-terminus, a middle region containing five PDZ domains and a tyrosine phosphatase domain at the C-terminus. The central finding of our study is that PTPL1 is associated with TAPP1 and, to a lesser extent, TAPP2. Our results indicated that the interaction is mediated mainly through the first PDZ domain of PTPL1 binding to the C-terminus of TAPP1/TAPP2. Importantly, we demonstrated that immunoprecipitation of endogenous TAPP1 results in co-immunoprecipitation of PTPL1 and vice versa, suggesting that this interaction is indeed physiological.

Recently it has been shown, in a breast cancer MCF7 cell line, that the overexpression of PTPL1 inhibited the activation of PI 3-kinase by inducing the dephosphorylation of an insulin-receptor-substrate protein [38]. In these cells 4-hydroxytamoxifen also induced apoptosis by inhibiting the activation of PI 3-kinase, an effect that was blocked by depleting PTPL1 from the cells with an antisense approach [38]. These results provide the first indication that the PI 3-kinase pathway could be a physiological target for the PTPL1 phosphatase. The findings of our study indicated that the binding of PTPL1 to TAPP1/TAPP2 could provide a mechanism by which PI 3-kinase could be down-regulated by PTPL1 and PtdIns(3,4)P_2. We propose that a pool of endogenous PTPL1 is maintained in the cytoplasm of unstimulated cells through its interaction with TAPP1. Following prolonged activation of PI 3-kinase, significant levels of PtdIns(3,4)P_2 accumulate at the membrane leading to the recruitment of the PTPL1–TAPP1 complex to this location. Here PTPL1 could dephosphorylate receptor(s) and/or adaptor protein(s), thereby inducing the inactivation of PI 3-kinase. Thus, PtdIns(3,4)P_2 could trigger the activation of a negative feedback loop to switch off PI 3-kinase. As mentioned in the introduction, some evidence also exists that *in vitro* TAPP1 may also interact with PtdIns(3,4,5)P_3, albeit more weakly than with PtdIns(3,4)P_2 [12]. Even if TAPP1 bound to PtdIns(3,4,5)P_3 *in vivo*, this would not compromise its ability to function as a negative inhibitor of the PI 3-kinase pathway once PtdIns(3,4,5)P_3 levels were elevated.

The finding that siRNA-mediated reduction in the expression of TAPP1 in HEK-293 cells moderately enhanced IGF1-mediated phosphorylation and activation of PKB, supports the role that TAPP1 exerts a negative influence on the PI 3-kinase pathway (Figure 9). PKB activation and phosphorylation is only moderately enhanced in TAPP1-knockdown cells following IGF1 stimulation. It is possible that the remaining 10–20 % levels of TAPP1 expression and/or expression of TAPP2 could be sufficient to maintain suppression of the PI 3-kinase pathway in cells.

Recent work has shown that the FERM domain of PTPL1 plays a crucial role in localizing full-length PTPL1 to the plasma membrane, which was suggested to be mediated through PtdIns(4,5)P_2 binding [35]. However, employing the same protein–lipid-overlay assay that was used previously to assess binding of PTPL1 to phosphoinositides [35], we were unable to demonstrate a significant interaction of full-length wild-type PTPL1 with PtdIns(4,5)P_2 (Figure 7, panel 1). Significantly we found that the interaction of PTPL1 with TAPP1 inhibited the

constitutive membrane localization of PTPL1, indicating that TAPP1 may sequester PTPL1 in the cytoplasm. This would keep PTPL1 away from the plasma membrane, which might otherwise dephosphorylate receptor and adaptor proteins to prevent activation of one or more components of signal transduction pathways. The mechanism by which TAPP1 sequesters PTPL1 in the cytosol requires further investigation. Following H_2O_2 treatment of cells, the levels of both PTPL1 and TAPP1 are recruited to the plasma membrane where PtdIns(3,4)P_2 would be located (Figure 6B). Consistent with this we demonstrated that, *in vitro*, PTPL1 complexed to TAPP1 can localize with PtdIns(3,4)P_2 spotted on to a membrane surface (Figure 7).

There is significant evidence that the PTP1B tyrosine phosphatase participates in the dephosphorylation of insulin receptor and therefore negatively regulates the insulin signalling pathway (reviewed in [39]). This is also confirmed by the finding that mice lacking PTP1B are significantly sensitized to insulin [40]. These discoveries have stimulated significant interest in developing inhibitors of PTP1B for the treatment of type 2 diabetes [41]. As, in tissues of mice lacking PTP1B, the insulin signalling pathway is still switched off following removal of insulin, other tyrosine phosphatases are also likely to be involved in dephosphorylating insulin receptor and insulin receptor substrate proteins [39]. The identity of these enzymes is unknown, but our results indicate that PTPL1 could play a role in negatively regulating the insulin-signalling pathway. If this can be substantiated by further research, it would indicate that inhibitors of PTPL1 or drugs that prevent PTPL1 from binding to TAPP1, could function to potentiate insulin-signalling pathways and could thus have therapeutic potential for the treatment of diabetes.

To our knowledge, the present study is the first to show that full-length PTPL1 possesses similar PNPP phosphatase activity to the isolated catalytic domain and we also demonstrate that the binding of TAPP1 to PTPL1 does not influence catalytic activity (Figure 8). This conflicts with the report of Nakai et al. [42], who claimed that the isolated catalytic domain of PTPL1 was 100-fold more active than the full-length protein. However, these authors did not show control experiments to establish that similar levels of full-length PTPL1 and catalytic domain were present in their assays. Moreover, in these studies, the full-length PTPL1 was expressed in HEK-293 cells, whereas the catalytic domain was expressed in *E. coli* [42]. Although our results indicate that the PDZ domains of PTPL1 do not influence the catalytic activity of PTPL1 directly, it is possible that they could play a key role in regulating the localization of PTPL1 and/or control its interaction with cellular substrates. PTPL1 has been reported to interact with numerous proteins through its PDZ domains including the Fas receptor [28,43], neurotrophin receptor [34], a bromodomain-containing protein BP75 [37], the adenomatous polyposis coli-protein [44], the transcription inhibitory protein IκBα [42,45], PKC-related kinase-2 [46] and the GTPase-activating protein PARG1 [47]. With the exception of Fas, these interactions have not been confirmed by co-immunoprecipitation of endogenous PTPL1 and the functional relevance of these interactions has not been defined. Our results indicate that TAPP1 is not phosphorylated on tyrosine residues in cells, as it is not recognized by anti-phosphotyrosine antibodies when expressed in unstimulated, IGF1- or pervanadate-treated cells (W. A. Kimber, unpublished work). Our results suggest that TAPP1 is more likely to function as a regulator of the cellular localization of PTPL1, allowing PTPL1 to translocate to the plasma membrane when the levels of PtdIns(3,4)P_2 are elevated.

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