Evidence that the tandem-pleckstrin-homology-domain-containing protein TAPP1 interacts with Ptd(3,4)P_2 and the multi-PDZ-domain-containing protein MUPP1 in vivo

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INTRODUCTION

Agonists, including growth factors, hormones and cytokines, induce the activation of members of the phosphoinositide 3-kinase (PI 3-kinase) group of lipid kinases, which phosphorylate PtdIns(4,5)_P_2, namely PtdIns(3,4)_P_2, whose levels are elevated by numerous extracellular agonists, might also function as a signalling molecule. Recently, we identified two related pleckstrin-homology-domain-containing proteins, termed ‘tandem-PH-domain-containing protein-1’ (TAPP1) and TAPP2, which interacted in vitro with high affinity with PtdIns(3,4)_P_2, but did not bind PtdIns(3,4,5)_P_3 or other phosphoinositides. In the present study we demonstrate that stimulation of Swiss 3T3 or 293 cells with agonists that stimulate PtdIns(3,4)_P_2 production results in the marked translocation of TAPP1 to the plasma membrane. This recruitment is dependent on a functional PtdIns(3,4)_P_2-binding PH domain and is inhibited by wortmannin, a phosphoinositide 3-kinase inhibitor that prevents PtdIns(3,4,5)_P_3 generation. A search for proteins that interact with TAPP1 identified the multi-PDZ-containing protein termed ‘MUPP1’, a protein possessing 13 PDZ domains and no other known modular or catalytic domains [PDZ is postsynaptic density protein (PSD-95)/Drosophila disc large tumour suppressor (dlg)/tight junction protein (ZO1)]. We demonstrate that immunoprecipitation of endogenously expressed TAPP1 from 293-cell lysates results in the co-immunoprecipitation of endogenous MUPP1, indicating that these proteins are likely to interact with each other physiologically. We show that TAPP1 and TAPP2 interact with the 10th and 13th PDZ domain of MUPP1 through their C-terminal amino acids. The results of the present study suggest that TAPP1 and TAPP2 could function in cells as adapter proteins to recruit MUPP1, or other proteins that they may interact with, to the plasma membrane in response to signals that elevate PtdIns(3,4)_P_2.

Key words: adapter protein, oxidative stress, phosphoinositide, phosphoinositide 3-kinase, scaffolding protein.

PtdIns(3,4,5)_P_2 is an established second messenger of growth-factor and insulin-induced signalling pathways. There is increasing evidence that one of the immediate breakdown products of PtdIns(3,4,5)_P_2, namely PtdIns(3,4)_P_2, whose levels are elevated by numerous extracellular agonists, might also function as a signalling molecule. Recently, we identified two related pleckstrin-homology-domain (PH)-domain-containing proteins, termed ‘tandem-PH-domain-containing protein-1’ (TAPP1) and TAPP2, which interacted in vitro with high affinity with PtdIns(3,4)_P_2, but did not bind PtdIns(3,4,5)_P_3 or other phosphoinositides. In the present study we demonstrate that stimulation of Swiss 3T3 or 293 cells with agonists that stimulate PtdIns(3,4)_P_2 production results in the marked translocation of TAPP1 to the plasma membrane. This recruitment is dependent on a functional PtdIns(3,4)_P_2-binding PH domain and is inhibited by wortmannin, a phosphoinositide 3-kinase inhibitor that prevents PtdIns(3,4,5)_P_3 generation. A search for proteins that interact with TAPP1 identified the multi-PDZ-containing protein termed ‘MUPP1’, a protein possessing 13 PDZ domains and no other known modular or catalytic domains [PDZ is postsynaptic density protein (PSD-95)/Drosophila disc large tumour suppressor (dlg)/tight junction protein (ZO1)]. We demonstrate that immunoprecipitation of endogenously expressed TAPP1 from 293-cell lysates results in the co-immunoprecipitation of endogenous MUPP1, indicating that these proteins are likely to interact with each other physiologically. We show that TAPP1 and TAPP2 interact with the 10th and 13th PDZ domain of MUPP1 through their C-terminal amino acids. The results of the present study suggest that TAPP1 and TAPP2 could function in cells as adapter proteins to recruit MUPP1, or other proteins that they may interact with, to the plasma membrane in response to signals that elevate PtdIns(3,4)_P_2.

Key words: adapter protein, oxidative stress, phosphoinositide, phosphoinositide 3-kinase, scaffolding protein.

INTRODUCTION

Agonists, including growth factors, hormones and cytokines, induce the activation of members of the phosphoinositide 3-kinase (PI 3-kinase) group of lipid kinases, which phosphorylate PtdIns(4,5)_P_2 at the D3 position of the inositol head group to generate PtdIns(3,4,5)_P_2 [1]. This lipid is located at the plasma membrane and functions as an important cellular ‘second messenger’, triggering the activation of a network of signalling pathways that regulate many physiological events, including cell survival and responses to insulin. A key mechanism by which PtdIns(3,4,5)_P_2 is known to activate downstream signal-transduction events is by its ability to interact specifically with members of a group of proteins that possess a certain type of pleckstrin-homology (PH) domain [2]. This interaction results in their recruitment to the plasma membrane, where they are brought into the vicinity of their physiological effectors and/or are activated by phosphorylation at this location (reviewed in [1,3]). PtdIns(3,4,5)_P_2 can be further metabolized to PtdIns(3,4)_P_2 through the action of 5-phosphatases termed Src-homology-domain-2-containing inositol 5-phosphatase-1 (SHIP1) and SHIP2 [4,5]. Several, but not all PH-domain-containing proteins that interact with PtdIns(3,4,5)_P_2, also bind to PtdIns(3,4)_P_2 with similar affinity. These include the PH domains of protein kinase B (PKB, also known as Akt) [6], 3-phosphoinositide-dependent protein kinase-1 (PKD1) [7,8] and the dual adaptor for phosphotyrosine and phosphoinositides-1 (DAPP1) [9–11]. Recent work has suggested that PtdIns(3,4)_P_2 can also be generated via a pathway involving the phosphorylation of PtdIns3P by an as yet-uncharacterized PI 4-kinase in response to cross-linking of platelet integrin receptors [12,13]. It has also been shown that treatment of several cell lines with H_2O_2 resulted in a large

Abbreviations used: ARF, ADP-ribosylation factor; BTK, Bruton’s tyrosine kinase; DAPP1, dual adaptor for phosphotyrosine and 3-phosphoinositides; EST, expressed sequence tag; FAPP1, PtdIns-four-phosphate adapter protein-1; GAP, GTPase-activating protein; GBD, Gal4-DNA-binding domain; GFP, green fluorescent protein; GRP1, general receptor for phosphoinositides-1; GST, glutathione S-transferase; IGF, insulin-like growth factor; MUPP1, multi-PDZ-domain protein-1 [where PDZ is postsynaptic density protein (PSD-95)/Drosophila disc large tumour suppressor (dlg)/tight junction protein (ZO1)]; PKC, protein kinase C; PDGF, platelet-derived growth factor; PKD1, 3-phosphoinositide-dependent protein kinase-1; PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; SD agar, synthetic dropout agar; SHIP, Src-homology-domain-2-containing inositol 5-phosphatase; TAPP, tandem-PH-domain-containing protein; TAPP1(R286L), etc., TAPP1 in which Arg286 has been mutated to Leu etc.; X-Gal, 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside; YFP, yellow fluorescent protein.

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increase in PtdIns(3,4)P₂ levels, with only a small and transient increase in PtdIns(3,4,5)P₃ [14]. The pathway by which PtdIns(3,4)P₂ is generated in response to H₂O₂ has not been characterized.

These findings raise the possibility that PtdIns(3,4)P₂ functions as a signalling lipid in vivo. Thus far only two related proteins of unknown physiological function, termed the tandem-PH-domain-containing protein-1 (TAPP1) and TAPP2, have been identified, which possess the ability to bind PtdIns(3,4,5)P₃ specifically. TAPP1 and TAPP2 consist of two sequential PH domains, of which the C-terminal one binds to PtdIns(3,4)P₂ with high affinity. In our hands the C-terminal PH domain of TAPP1 does not interact significantly with PtdIns(3,4,5)P₃ or any other phosphoinositide that has been tested [15]. The three-dimensional structure of the C-terminal PH domain of TAPP1 has recently been determined, establishing the mechanism by which it interacts specifically with PtdIns(3,4)P₂ [16]. It should be noted, however, that Lemmon and colleagues [11] have reported that the C-terminal PH domain of TAPP1 interacts weakly with Ins(1,3,4,5)P₄, the water-soluble head group of PtdIns(3,4,5)P₃, raising the possibility that, in vivo, TAPP1 could potentially bind Ins(1,3,4,5)P₄ or PtdIns(3,4,5)P₃. In the present study we provide evidence that TAPP1 interacts specifically with PtdIns(3,4)P₂ in vivo, resulting in its recruitment to the plasma membrane. Furthermore, we show that TAPP1 and TAPP2 interact through their C-terminal residues with a multi-PDZ-containing protein termed MUPP1 [17].

MATERIALS AND METHODS

Materials

Protease-inhibitor-cocktail tablets, FuGENE™ transfection reagent and G418 (Geneticin) were from Roche, insulin-like growth factor-1 (IGF1) and microcin-LR (a cyanobacterial peptide toxin) were from Life Technologies, foetal bovine serum and other tissue-culture reagents were from BioWhittaker, dimethyl pimelimidate and H₂O₂ were from Sigma, and platelet-derived growth factor (PDGF)-BB was from Calbiochem. The precast 4–12 % bis-Tris gradient gels were from Invitrogen.

Antibodies

Antibodies recognizing TAPP1 were raised in sheep against the whole TAPP1 protein expressed in Escherichia coli as a glutathione S-transferase (GST) fusion. The antibodies recognizing MUPP1 were raised against a C-terminal fragment of MUPP1 comprising residues 926–1676 expressed in E. coli as a GST-fusion protein and are available from Upstate Biotechnology. The anti-LKB1 antibody used as a negative control was raised against the whole LKB1 (a serine/threonine kinase) protein [18]. The antibodies were affinity-purified on CH-Sepharose covalently coupled to the protein antigens used to raise the antibodies. The anti-TAPP1 and anti-MUPP1 antibodies were then passed through a column of CH-Sepharose coupled to GST and the antibody that did not bind was selected. Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were from Pierce, monoclonal antibodies recognizing GST and Flag epitope tags were from Sigma.

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. This was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Dundee, Scotland, U.K., using DYEnamic ET terminator chemistry (Amersham Pharmacia Biotech) 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, and 0.1 mM EGTA. Buffer C contained 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 % (w/w) Triton X-100, 1 mM Na₂VO₄, 50 mM NaF, 5 mM sodium pyrophosphate (Na₂H₂P₂O₇), 0.27 M sucrose, 1 µm microcystin-LR, 0.1 % (v/v) 2-mercaptoethanol and ‘complete’ protease-inhibitor cocktail (one tablet/25 ml). Sample Buffer contained 50 mM Tris/HCl, pH 6.8, 2 % (w/w) SDS, 10 % (v/v) glycerol and 1 % (v/v) 2-mercaptoethanol.

DNA constructs

Mammalian fusions GST–TAPP1 and GST–TAPP2 in the pEBG-2T expression vector have been described previously [15]. The FLAG-epitope-tagged full-length human TAPP1 was amplified by PCR using the primers N1 (5'-GGATCCGCCACCATG-GACTCAAGGACAGCATGACAAATGCTGGTGTGCTGCAATCCG-3') and N2 (5'-GGATCCACGAATTCAGGATCTGATGTTTTGCCTG-3'), with the primer TAPP1 cDNA and 0.1 mM EGTA. Buffer D contained 50 mM Tris

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GST-tagged MUPP1 fragments employed in the experiments described in Figure 5 (below), BgII–BgII fragments derived from pACT2 TAPP1 interacting two-hybrid clones were subcloned into the BamH1 site of pEBG-3X vector. The constructs of MUPP11–13 fragment comprise amino acids 1689–2042 and those of the MUPP12 fragment comprise amino acids 1885–2042. The MUPP11–13 fragment, comprising amino acids 1689–1924, was generated by introducing a STOP mutation in the MUPP11–13 pEBG2T construct.

**Cell culture, stimulation and cell lysis**

Mouse fibroblast Swiss 3T3 and human embryonic kidney 293 cells were cultured on 10 cm-diameter dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) foetal calf serum. Where indicated the cells were cultured in the absence of serum. Wortmannin was dissolved in DMSO at a 1000-fold higher concentration than that at which it was used. It, or the equivalent volume of DMSO as a control, was added to the tissue-culture medium 10 min prior to stimulation. The cells were stimulated with the indicated agonists and either fixed in 4% (v/v) paraformaldehyde or lysed in 1 ml of ice-cold Buffer C. In the latter case the lysates were centrifuged at 4 °C for 5 min at 16000 g. The supernatants were frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined using the Bradford method, and BSA was employed as the standard.

**Generation of stable Swiss 3T3 cell lines expressing YFP and YFP–TAPP1ct**

Swiss 3T3 cells were cultured to 50% confluence on 10 cm-diameter dishes and transfected with 2.5 μg of the pEYFP–TAPP1ct or empty pEYFP vector using FuGENE™ 6 transfection reagent following the manufacturer’s (Roche) protocol. A triplicate set of dishes was used for each condition. After 48 h, G418 was added to the medium to a final concentration of 3 mg/ml, and the medium was changed every 48 h, maintaining G418. After 10 days, colonies of fluorescently cells were isolated and expanded. We selected a cell line for analysis that expressed an intermediate level of YFP or YFP–TAPP1ct.

**Microscopy and image analysis**

In experiments in which the cells were fixed prior to analysis, the cells were grown on coverslips (no. 1) and after stimulation were washed in PBS and fixed for 10 min in freshly prepared 4% (v/v) paraformaldehyde in PHEM Buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA and 2 mM MgSO4, pH 7.0). The cells were washed twice with PBS and mounted on to slides using Mowiol. For live-cell imaging the cells were grown on 42 mm-diameter glass coverslips. Cells were maintained at 37 °C by the use of a closed perfusion chamber (Bioptechs, Butler, PA, U.S.A.) FCS2. Images were collected using the 100 ×-magnification 1.4-numerical aperture Apochromat objective on a Delta Vision–Zeiss restoration microscope. For each cell, 20 optical sections separated by 0.5 μm were recorded, using a Sony Micromax CCD (charge-coupled device) camera and an effective pixel size of 0.265 × 0.265 μm. In all cases the exposure time was 100 ms and the mercury-lamp excitation light was attenuated with a 0.3 neutral-density filter. Three-dimensional images were recorded every 90 s over a period of 15 min for each condition. The three-dimensional motorized stage allowed the imaging of six cells in each experiment. Images were corrected for any fluctuations in mercury-lamp power and restored by iterative constrained deconvolution using an empirically measured point spread function [20]. Time-lapse images were viewed as single section projections of each time point. All these manipulations were performed using routines contained within the softWoRx image processing package (Applied Precision Inc., Issaquah, WA, U.S.A.).

**Immunoelectron microscopy**

Cells were cultured on a 10 cm-diameter plastic dishes to 75% confluency and the medium removed before addition of 8% (w/v) paraformaldehyde in 0.2 M Pipes, pH 7.2, for 30 min at room temperature, followed by at least 2 days at 4 °C. Cells were then removed from the dish with a plastic cell scraper and pelleted in fixative at approx. 10000 g for 30 min. After cryo-protection in 2.1 M sucrose in PBS, ultrathin sections were cut at approx. −100 °C in a Leica ultracryomicrotome and mounted on Piooloform® (polyvinylformal)/carbon-coated grids. Sections were retrieved using the modified pick-up method of Liou et al. [21] and labelled at room temperature as follows. Grids were placed on drops of 0.5% fish skin gelatin in PBS for 10 min and transferred to drops of rabbit anti-green fluorescent protein (GFP) antibody (a gift from Dr David Shima, Endothelial Cell Biology Laboratory, Imperial Cancer Research Fund Laboratories, London, U.K.) for 30 min. Following washes in PBS (3 × 5 min) the grids were incubated on Protein A–gold (8 nm particle size [22]) for 20 min, washed first in PBS (6 × 5 min) and then distilled water (10 × 1 min). The sections were contrasted in methylcellulose/uranyl acetate [23] and observations and photographs taken on a JEOL 1200EX electron microscope. For quantification, micrographs were taken at systematically spaced locations (with a random start) at a primary magnification of 15000 ×. The negatives were scanned at a resolution of 1000 pixels/in (≈ 394 pixels/cm), displayed in Adobe Photoshop 5.5 and square-lattice grids superimposed electronically at a line spacing of 0.5 μm. The length of the plasma membrane and the area of cytoplasm were estimated using principles and formulae described previously [24]. Labelling density of lamellipodia-like protrusions and plasma membrane was compared by systematically scanning immunolabelled cell pellet profiles at a primary magnification of 20000 ×. Intersections of a ‘scanning line’ defined by a feature on the viewing screen [25] and gold particle labelling observed during scanning were assigned to either lamellopodia or plasma membranes. The estimates of gold density per intersection were calculated as ratio estimates after at least ten scans per pellet profile.

**Yeast two-hybrid screen**

Myc-tagged human TAPP1 was subcloned into the EcoR1/SalI site of pAS2-1 (Clontech) as a Gal4-DNA-binding domain fusion (GBD). A yeast two-hybrid screen was carried out by co-transforming pAS2-1 TAPP1 and a pACT2 human brain cDNA library fused to the Gal4 activation domain into the yeast strain Y190. The brain library was purchased from Clontech. Transformed yeast cells were incubated for 10 days at 30 °C on synthetic dropout (SD) agar supplemented with 25 mM 3-aminoitolzazole and lacking histidine, leucine and tryptophan. Approx. 1 × 106 colonies were screened. A total of 140 colonies, which grew on media lacking histidine, leucine and tryptophan, were picked and assayed for expression of the lacZ reporter gene, which codes for β-galactosidase. All 140 colonies tested positive in the 5-bromo-4-chloroindol-3-yl β-(methylthio)galactopyranoside (X-Gal) assay. The pACT2 plasmids from these clones were isolated and the inserts sequenced using standard procedures in order to identify candidate TAPP1 interacting proteins.
Figure 1  Live-cell imaging of the translocation of YFP–TAPP1CT to the plasma membrane in response to H$_2$O$_2$ and PDGF

Serum-starved Swiss 3T3 cells stably expressing YFP–TAPP1CT were mounted in a heated chamber for live cell fluorescent imaging using a Delta Vision–Zeiss restoration microscope. Images were collected every 90 s. (A and C) Serum-starved cells were visualized for 15 min, and the panel labelled ‘Control’ corresponds to the image of the cell after this period. At this point the medium of the cells was exchanged with pre-warmed medium containing either 1 mM H$_2$O$_2$ (A) or 50 ng/ml PDGF (C) and the cells were observed for a further 15 min, and an image of the cells after 3 and 15 min is shown. (B and D) As above, except that, after imaging the serum-starved cells for 15 min, the medium of the cells was exchanged with pre-warmed medium containing 100 nM wortmannin and the cells were left incubating with this drug for a further 15 min. The image termed ‘+ Wortmannin’ corresponds to the cells after this period. The medium was then exchanged with pre-warmed medium containing 100 nM wortmannin and either 1 mM H$_2$O$_2$ (B) or 50 ng/ml PDGF (D) and the cells were observed for a further 15 min. The image of the cell after this period is shown. (E and F) Serum-starved Swiss 3T3 cells transiently transfected with a construct expressing YFP–TAPP1CT[R211L] were either left unstimulated or stimulated with 1 mM H$_2$O$_2$ (E) or 50 ng/ml PDGF for 15 min (F). For all panels, each experiment was performed at least in duplicate and six separate cells were visualized in each experiment. A representative cell from these experiments is shown. Arrows indicate accumulation of YFP–TAPP1CT at the plasma membrane. The scale bars represent 10 μm. An animated time-lapse version of each panel can be viewed in Movies 1A–1F, which are downloadable from the website given in the text.
Yeast two-hybrid analysis of the interaction of TAPP1/TAPP2 with MUPP1

For the analysis shown in Figure 4 (below), Y190-strain yeasts were co-transformed with the indicated combinations of vectors and grown on SD agar lacking tryptophan and leucine at 30 °C until appearance of colonies. Yeast colonies were picked and resuspended in 50 μl of sterile water and 1 μl dropped on to SD agar lacking leucine and tryptophan or SD agar supplemented with 25 mM 3-amino triazole and lacking histidine, leucine and tryptophan. The yeast patches were incubated for 2 days at 30 °C and filter lifts taken of yeast grown on SD agar lacking leucine and tryptophan. Reporter β-galactosidase activity of the transformants was tested by incubating filters in X-Gal at 30 °C for 4 h.

Expression of GST–MUPP1 fusion proteins in E. coli

The pGEX-2T constructs encoding GST–MUPP1 fragments were transformed into BL21 E. coli cells and a 0.5 litre culture was grown at 37 °C in Luria broth containing 100 μg/ml ampicillin, until the A600 was 0.6. Isopropyl β-D-galactosidase (250 μM) was added and the cells cultured for a further 16 h at 26 °C. The cells were resuspended in 25 ml of ice-cold Buffer C and lysed by one round of freeze–thawing and the lysates sonicated to fragment the DNA. The lysates were centrifuged at 4 °C for 30 min at 20000 g, the supernatant filtered through a 0.44 μm-pore-size filter and incubated for 60 min on a rotating platform with 1 ml of GSH–Sepharose previously equilibrated in Buffer C. The suspension was centrifuged for 1 min at 3000 g, the beads washed three times with 15 ml of Buffer C containing 0.5 M NaCl, and then a further ten times with 15 ml of Buffer A. The GST–Sepharose in an equal volume of Buffer A was divided and the cells cultured for a further 16 h at 26 °C. The cells were resuspended in 25 ml of ice-cold Buffer C and lysed by one round of freeze–thawing and the lysates sonicated to fragment the DNA. The lysates were centrifuged at 4 °C for 30 min at 20000 g, the supernatant filtered through a 0.44 μm-pore-size filter and incubated for 60 min on a rotating platform with 1 ml of GSH–Sepharose previously equilibrated in Buffer C. The suspension was centrifuged for 1 min at 3000 g, the beads washed three times with 15 ml of Buffer C containing 0.5 M NaCl, and then a further ten times with 15 ml of Buffer A. The GST–Sepharose in an equal volume of Buffer A was divided into aliquots, snap-frozen in liquid nitrogen, and stored at −80 °C. This was employed for the binding assays described in Figure 6 (below).

Binding of wild-type and mutant TAPP1/TAPP2 to MUPP1 fragments in 293 cells

For the results presented in Figure 5 (below), 293 cells were co-transfected with 5 μg of the wild-type or mutant FLAG-epitope-tagged TAPP1/TAPP2 pCMV5 plasmids and 5 μg of the pEBG2T plasmids encoding the indicated GST–MUPP1 C-terminal fragments. At 36 h post-transfection the cells were lysed in 1 ml of Buffer C, the lysates were cleared by centrifugation at 13000 g for 10 min at 2 °C, and 0.5 mg of supernatant was incubated for 1 h at 4 °C with 5 μl of GSH–Sepharose. The beads were washed twice in Buffer C containing 0.15 M NaCl, followed by two further washes in Buffer A. The beads were resuspended in 20 μl of Sample Buffer and subjected to SDS/PAGE. The gels were analysed by immunoblotting with either anti-FLAG or anti-GST antibodies (described below).

Expression of wild-type and mutant GST–TAPP1 and GST–TAPP2 in human embryonic kidney 293 cells

For purification of GST-fusion proteins, shown in Figure 7(B) below, 10-cm-diameter dishes of 293 cells were transfected with 5 μg of the indicated pEBG-2T construct encoding wild-type or mutant TAPP1 and TAPP2, using a modified calcium phosphate method [26]. At 36 h post-transfection the cells were lysed in 1 ml of Buffer C, the lysates were cleared by centrifugation at 13000 g for 10 min at 2 °C, and 2 mg of supernatant was incubated for 1 h at 4 °C with 10 μl of GSH–Sepharose. The GST-fusion proteins were purified by affinity chromatography and analysed as described in the legend to Figure 7(B) below.

Immunoprecipitation of endogenous TAPP1 and MUPP1

The polyclonal anti-TAPP1, anti-MUPP1 or anti-LKB1 antibodies (0.5 mg) were covalently coupled to Protein G–Sepharose (0.5 ml) using dimethyl pimelimidate [27]. 293-cell lysates (3 mg) were incubated for 60 min at 4 °C with the Protein G–Sepharose conjugates (15 μl). The immunoprecipitates were washed twice with 1 ml of Buffer C containing 0.15 M NaCl, and washed twice with Buffer B. The beads were resuspended in SDS Sample Buffer that did not contain 2-mercaptoethanol and analysed as described in the legend to Figure 7(A) below.

Immunoblotting

For blots of total cell lysates, 10–20 μg of protein was used. For blots of GST pull-downs or immunoprecipitation, the entire amount of beads was used. 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 % w/v Tween (TBS-Tween)/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 horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL*: Amer sham Pharmacia Biotech) reagent.

RESULTS

Subcellular localization of the PtdIns(3,4)2-binding PH domain of TAPP1

We employed mouse fibroblast Swiss 3T3 cells as a model cell line to investigate whether TAPP1 could interact with PtdIns(3,4)2, in vivo, since the levels of PtdIns(3,4)2 and PtdIns(3,4,5)P3 generated in response to several stimuli have been well characterized in these cells. For example, stimulation of Swiss 3T3 cells with H2O2 (1 mM) induces a > 30-fold elevation in PtdIns(3,4)2, whilst only transiently increasing PtdIns(3,4,5)P3 levels, which returned to basal levels within 15 min. Stimulation with PDGF (50 ng/ml for 10 min), in contrast, induces a moderate increase in PtdIns(3,4)2, to 30 % of the level observed with H2O2, but increases basal PtdIns(3,4,5)P3 levels > 20-fold [14,28]. To perform our analysis, we generated Swiss 3T3 cells that stably express the PtdIns(3,4)2-binding PH domain of TAPP1 with an N-terminal YFP tag (YFP–TAPP1CT). Fluorescence time-lapse microscopy of living cells revealed that, in unstimulated, Swiss 3T3 cells, YFP–TAPP1CT was diffusely localized in the cytosolic and nuclear compartments. However, stimulation of these cells with H2O2 or PDGF induced a rapid recruitment of YFP–TAPP1CT to the plasma membrane, which was apparent within 3 min and PtdIns(3,4)2 remained located at the membrane for 15 min [Figures 1A and 1C; Movies 1A and 1C (these and subsequently cited animated time-lapse movies are downloadable from http://www.Biochemj.org/bj/361/bj3610525add.htm)]. Pre-treatment of cells with wortmannin prior to stimulating with H2O2 or PDGF prevented the translocation of YFP–TAPP1CT to the plasma membrane (Figures 1B and 1D; Movies 1B and 1D). Similar results were obtained in numerous separate experiments that were performed on unstimulated, H2O2- or PDGF-stimulated Swiss 3T3 cells that had been fixed in paraformaldehyde prior to analysing the location of YFP–TAPP1CT by fluorescence microscopy (results not shown). Similar results were also obtained when YFP–TAPP1CT was expressed in Swiss 3T3 cells by transient transfection (results not shown). In Figures 1(E) and 1(F) (and Movies 1E and 1F), we demonstrate that a mutant of TAPP1 that does not interact with PtdIns(3,4)2, YFP–TAPP1CT[R211L],...
was not recruited to the plasma membrane when Swiss 3T3 cells were stimulated with H$_2$O$_2$ and PDGF. As a further control we generated Swiss 3T3 cells that stably express YFP alone, which was found to be localized diffusely throughout the cytosol and nucleus in unstimulated, H$_2$O$_2$- and PDGF-stimulated cells (results not shown). We also generated human embryonic kidney 293 cells and human glioma U87MG cells, which stably expressed YFP–TAPP$_{1\text{ct}}$, and found that exposure of these cells to H$_2$O$_2$ induced a very marked translocation of YFP–TAPP$_{1\text{ct}}$ to the plasma membrane that was inhibited by wortmannin (results not shown).

**Immunogold localization of TAPP1**

The subcellular distribution of YFP–TAPP$_{1\text{ct}}$ was investigated using quantitative immunoelectron microscopy on ultrathin cryosections (Figures 2A–2D). In unstimulated Swiss 3T3 cells there was substantial immunolabelling for YFP–TAPP$_{1\text{ct}}$ over the cytosol, with little evidence for plasma-membrane localization or localization to intracellular organelles. In contrast, after H$_2$O$_2$ and PDGF stimulation, marked labelling of the plasma membrane was observed and, in the case of PDGF, this appeared most concentrated over extended cell protrusions that most likely represented lamellipodia (Figure 2D). This was quantified as described in the Materials and methods section, and it was estimated that there was a 1.9- and 1.7-fold increase in two separate experiments in the labelling concentration in these lamellipodia-like structures derived from PDGF-stimulated cells compared with the rest of the plasma membrane. Further quantitative analysis revealed that, compared with unstimulated cells, H$_2$O$_2$ and PDGF produced 10.4- and 5.2-fold overall accumulation of YFP–TAPP$_{1\text{ct}}$ at the cell surface and also showed that translocation to the plasma membrane was largely prevented by pre-incubation of cells with 100 nM wortmannin prior to stimulation with PDGF and H$_2$O$_2$ (Figure 2E).

**Evidence that TAPP1 does not interact with PtdIns(3,4,5)$_P$ in vivo**

We next decided to investigate whether stimulation of Swiss 3T3 cells with an agonist that generated PtdIns(3,4,5)$_P$, but only low levels of PtdIns(3,4)$_P$, would induce recruitment of YFP–TAPP$_{1\text{ct}}$ to the plasma membrane. IGF1 stimulation of Swiss 3T3 cells was shown previously to induce a >10-fold increase in PtdIns(3,4,5)$_P$ over the basal levels, but to only marginally elevate PtdIns(3,4)$_P$ [14]. Consistent with TAPP1 only interacting with PtdIns(3,4)$_P$, stimulation of Swiss 3T3 cells expressing YFP–TAPP$_{1\text{ct}}$ with IGF1 for up to 15 min did not result in a detectable recruitment of TAPP1 to the plasma membrane (Figure 3A; Movie 3A). As a control we showed that IGF1 stimulation of Swiss 3T3 cells induces translocation to the plasma membrane of the YFP-tagged PH domain of GRP1 (Figure 3B; Movie 3B), which binds PtdIns(3,4,5)$_P$ but not PtdIns(3,4)$_P$ [11,29]. IGF1 also induced recruitment to the plasma membrane of the GFP-tagged PH domain of PKB that binds both PtdIns(3,4,5)$_P$ and PtdIns(3,4)$_P$ (Figure 3C). IGF1, as expected, failed to induce any movement of the isolated YFP expressed in Swiss 3T3 cells (results not shown).

**Interaction of TAPP1 and TAPP2 with MUPP1**

In order to identify proteins that interact with TAPP1, we performed a yeast two-hybrid screen in which a human brain library was screened with full-length TAPP1 as the bait. In all, 35 out of the 140 TAPP1-binding ‘positive clones’ that were characterized corresponded to different C-terminal fragments of...
In vivo localization of PtdIns(3,4)_2^1

Figure 3  Live-cell imaging of YFP–TAPP1_CT, YFP–GRP1 and GFP–PKB in unstimulated and IGF1-stimulated Swiss 3T3 cells

Serum-starved Swiss 3T3 cells stably expressing YFP–TAPP1_CT were mounted in a heated chamber for live-cell fluorescent imaging using a Delta Vision–Zeiss restoration microscope. Images were collected every 90 s.  (A) Serum-starved cells were visualized for 15 min and the panel labelled ‘Control’ corresponds to the image of the cell after this period. At this point the medium was exchanged with pre-warmed medium containing 100 ng/ml IGF1 and the cells were observed for a further 15 min; an image after 3 and 15 min stimulation is shown.  (B) As above, except that the Swiss 3T3 cells were transiently transfected with a construct expressing the PH domain of YFP–GRP1. At 8 h post-transfection, the cells were serum starved for 16 h prior to imaging.  (C) Swiss 3T3 cells were transiently transfected with a DNA construct encoding the GFP–PH domain of PKBα and either left unstimulated or stimulated with IGF1 for 10 min in the presence or absence of wortmannin as described above. The cells were fixed with 4% paraformaldehyde and imaged on a Leica microscope as described in the Materials and methods section. For the (A) panels, TAPP1_CT failed to translocate to the plasma membrane in response to IGF1 in three separate experiments performed on different days. For experiments performed in (A) and (B), six separate cells were visualized in each experiment. A representative cell from these experiments is shown. Experiments in (C) were performed twice with similar results. Arrows indicate accumulation of YFP–GRP1–PH domain and GFP–PKB–PH domain at the plasma membrane. The scale bars represent 10 μM. An animated time-lapse version of each panel can be viewed in Movies 3A and 3B, which are downloadable from the website given in the text.

the 218 kDa multi-PDZ-containing protein termed MUPP1, which possesses 13 sequential PDZ domains and no known catalytic domain. Inspection of the amino acid sequences of TAPP1 and TAPP2 revealed that they are 58 % identical over the first 300 amino acids, a region which encompasses both of the PH domains. However, there is no significant similarity between the C-terminal 100 residues of TAPP1 and TAPP2, except that seven out of the 11 extreme C-terminal amino acids of TAPP1 and TAPP2 are identical (Figure 4D). Inspection of the last three residues of TAPP1 and TAPP2 (Ser-Asp-Val), reveal that these conform to the minimal sequence motif required for binding to a PDZ domain ([ser/thr/Xaa-Val]/ile [30,31]). This suggested that TAPP1, as well as TAPP2, might interact through their C-termini with a PDZ domain of MUPP1. The only PDZ domain that was present on all of the MUPP1-interacting clones isolated from the yeast two-hybrid screen (Figure 4) or a mammalian human 293 cell co-expression assay (Figure 5). Both of these assays revealed that wild-type TAPP1 and TAPP2, but not TAPP1ΔCT or TAPP2ΔCT, interacted with MUPP111–13 or MUPP113–13 confirming that the C-terminal residues of TAPP1 and TAPP2 had the intrinsic ability to interact with at least the 13th PDZ domain of MUPP1. We next tested whether nine fragments of MUPP1 that encompass all 13 PDZ domains were able to bind TAPP1, TAPP1ΔCT, TAPP2 and TAPP2ΔCT (Figure 6). This analysis revealed that fragments of MUPP1 overlapping PDZ domains 1–9 or the isolated 11th or 12th PDZ domain failed to bind to TAPP1 or TAPP1 and MUPP1. We therefore initially tested whether wild-type TAPP1, TAPP2 or mutants of TAPP1 and TAPP2 that lack the six C-terminal amino acids (TAPP1ΔCT and TAPP2ΔCT) could interact with a fragment of MUPP1 encompassing only the 13th PDZ domain (MUPP113) or the 11–13th PDZ domain (MUPP111–13). We tested these interactions in a yeast two-hybrid screen (Figure 4) or a mammalian human 293 cell co-expression assay (Figure 5). Both of these assays revealed that wild-type TAPP1 and TAPP2, but not TAPP1ΔCT or TAPP2ΔCT, interacted with MUPP111–13 or MUPP113–13 confirming that the C-terminal residues of TAPP1 and TAPP2 had the intrinsic ability to interact with at least the 13th PDZ domain of MUPP1. We next tested whether nine fragments of MUPP1 that encompass all 13 PDZ domains were able to bind TAPP1, TAPP1ΔCT, TAPP2 and TAPP2ΔCT (Figure 6). This analysis revealed that fragments of MUPP1 overlapping PDZ domains 1–9 or the isolated 11th or 12th PDZ domain failed to bind to TAPP1 or TAPP1 and MUPP1. We therefore initially tested whether wild-type TAPP1, TAPP2 or mutants of TAPP1 and TAPP2 that lack the six C-terminal amino acids (TAPP1ΔCT and TAPP2ΔCT) could interact with a fragment of MUPP1 encompassing only the 13th PDZ domain (MUPP113) or the 11–13th PDZ domain (MUPP111–13). We tested these interactions in a yeast two-hybrid screen (Figure 4) or a mammalian human 293 cell co-expression assay (Figure 5). Both of these assays revealed that wild-type TAPP1 and TAPP2, but not TAPP1ΔCT or TAPP2ΔCT, interacted with MUPP111–13 or MUPP113–13 confirming that the C-terminal residues of TAPP1 and TAPP2 had the intrinsic ability to interact with at least the 13th PDZ domain of MUPP1. We next tested whether nine fragments of MUPP1 that encompass all 13 PDZ domains were able to bind TAPP1, TAPP1ΔCT, TAPP2 and TAPP2ΔCT (Figure 6). This analysis revealed that fragments of MUPP1 overlapping PDZ domains 1–9 or the isolated 11th or 12th PDZ domain failed to bind to TAPP1 or
GST–TAPP1 and GST–TAPP2 expressed in 293 cells and the immunoprecipitation procedure. We also demonstrate that of the TAPP1–MUPP1 complex occurs during either cell lysis or however, we cannot exclude the possibility that some dissociation that not all of the cellular MUPP1 is associated with TAPP1. antibody than with a TAPP1 antibody (Figure 7A), indicating precipitated from the same amount of cell lysate using a MUPP1 be noted that about 10-fold more MUPP1 can be immuno- immunoprecipitate TAPP1 did not pull down MUPP1. It should results in the co-immunoprecipitation of endogenous MUPP1. As a control we demonstrated that an antibody that does not recognize endogenously expressed MUPP1 and TAPP1. We is physiologically significant, we generated antibodies that could recognize the expression of HIS3 reporter gene, enabling growth on SD medium lacking leucine, SD medium without leucine and tryptophan, in which only those harbouring both pAS2-1 and pACT2 plasmids grow. An interaction between TAPP1/TAPP2 and MUPP111–13/MUPP113 leads (results not shown). We show in Figure 7 that the interaction of TAPP1 with MUPP1 (Figure 7B). The purified proteins were electrophoresed on an SDS/4–12%-(w/v)-polyacrylamide gel, and immunoblotted using either an anti-FLAG antibody to detect FLAG–TAPP1 or FLAG–TAPP2 or an anti-GST antibody to detect expression of GST-MUPP1 fusion proteins. To establish that the wild-type and mutant TAPP1 and TAPP2 forms were expressed at similar levels, 10 μg of total 293-cell lysate (termed ‘total lysate’) for each condition were electrophoresed on an SDS/4–12%-polyacrylamide gel and immunoblotted with anti-FLAG antibodies. Similar results were obtained in two different experiments. The positions of the molecular-mass markers (Bio-Rad Precision Markers) are indicated.

To investigate whether the interaction of TAPP1 and MUPP1 is physiologically significant, we generated antibodies that could recognize endogenously expressed MUPP1 and TAPP1. We decided to use human 293 cells in the experiments described below, because these cell lines expressed the highest levels of endogenous MUPP1 and TAPP1 among those that we investigated (results not shown). We show in Figure 7 that the immunoprecipitation of endogenous TAPP1 from 293-cell lysates results in the co-immunoprecipitation of endogenous MUPP1. As a control we demonstrated that an antibody that does not immunoprecipitate TAPP1 did not pull down MUPP1. It should be noted that 10-fold more MUPP1 can be immunoprecipitated from the same amount of cell lysate using a MUPP1 antibody than with a TAPP1 antibody (Figure 7A), indicating that not all of the cellular MUPP1 is associated with TAPP1. However, we cannot exclude the possibility that some dissociation of the TAPP1–MUPP1 complex occurs during either cell lysis or the immunoprecipitation procedure. We also demonstrate that GST–TAPP1 and GST–TAPP2 expressed in 293 cells and affinity-purified from cell lysates using GSH–Sepharose is associated with endogenously expressed MUPP1. In contrast, GST–TAPP1ACT and GST–TAPP2ACT did not interact with endogenous MUPP1 (Figure 7B).

Recruitment of full-length TAPP1 to the plasma membrane requires only the PtdIns(3,4)₂-binding PH domain

To determine whether the PDZ-binding C-terminus of TAPP1 or its N-terminal PH domain influenced the subcellular localization of TAPP1, 293 cells were transfected with full-length wild-type YFP–TAPP1, YFP–TAPP1ACT, TAPP1 possessing a mutation that would disrupt the N-terminal PH domain (YFP–TAPP1[R28L]) or the non-PtdIns(3,4)₂-binding mutant YFP–TAPP1[R211L]. As observed previously for the isolated C-terminal PtdIns(3,4)₂-binding PH domain (Figure 1), full-length TAPP1 was localized in the cytosol of unstimulated cells, and H₂O₂ induced its translocation to the plasma membrane, which was prevented by treatment of the cells with Wortmannin (Figure 8A). The translocation of full-length TAPP1 to membranes was dependent on the interaction of TAPP1 with PtdIns(3,4)₂, but not on its PDZ-binding motif, as the YFP–
The cellular location of PtdIns(3,4,5)$\_i$ only poorly induced the recruitment of these mutants of TAPP1 to the plasma membrane, indicating that they interact poorly with PtdIns(3,4,5)$\_i$ in vivo. The reasons for this are unclear, but, because of this finding, we cannot definitively rule out the possibility that TAPP1 may also interact with PtdIns(3,4)$\_o$, or any other phosphoinositide, that was tested [32]. Consistent with this observation, the N-terminal PH domain mutant of TAPP1, YFP–TAPP1[R28L] is recruited to cell membranes by H$_2$O$_2$ as effectively as wild-type TAPP1 (Figure 8B).

**DISCUSSION**

The present results indicate that PtdIns(3,4)$\_i$ is likely to be a physiological ligand for the C-terminal PH domain of TAPP1. This is based on the finding that stimulation of Swiss 3T3 cells with H$_2$O$_2$ or PDGF (Figures 1 and 2), 293 cells with H$_2$O$_2$ (Figure 8) and U87MG cells with H$_2$O$_2$ (results not shown), induces the marked translocation of TAPP1 to the plasma membrane, where PtdIns(3,4)$\_i$ would be expected to be located (see below). Consistent with translocation of TAPP1 to the plasma membrane being mediated through its interaction with PtdIns(3,4)$\_i$, it is prevented by wortmannin, a compound that inhibits PtdIns(3,4)$\_i$ production, or by mutation of a conserved arginine residue that is required for the interaction of TAPP1 with PtdIns(3,4)$\_i$ (Figures 1, 2 and 8). In contrast, stimulation of Swiss 3T3 cells with IGF1, an agonist that elevates PtdIns(3,4,5)$\_i$ without markedly increasing PtdIns(3,4)$\_i$, induced the translocation to the plasma membrane of the GRP1 PH domain that binds PtdIns(3,4,5)$\_i$ specifically or the PH domain of PKB that binds both PtdIns(3,4,5)$\_i$ and PtdIns(3,4)$\_i$, but failed to recruit TAPP1 (Figure 3). If Ins(1,3,4)$\_o$ or Ins(1,3,4,5)$\_o$ were physiological ligands for TAPP1, one would expect TAPP1 not to translocate to the plasma membrane in response to H$_2$O$_2$ or PDGF, as these inositol phosphate species would not be expected to be located at the plasma membrane. We also expressed two mutants of TAPP1, YFP–TAPP1[A203G,V204G] and YFP–TAPP1[A203G], which interact with both PtdIns(3,4)$\_i$ and PtdIns(3,4,5)$\_i$, in Swiss 3T3 cells in order to verify whether IGF1 stimulation of these cells induced translocation of this mutant to the plasma membrane. Unfortunately, in numerous experiments, IGF1 did not induce a translocation of these mutants to the plasma membrane (results not shown). Even H$_2$O$_2$ only poorly induced the recruitment of these mutants of TAPP1 to the plasma membrane, indicating that they interact poorly with PtdIns(3,4)$\_i$ in vivo. The reasons for this are unclear, but, because of this finding, we cannot definitively rule out the possibility that TAPP1 may also interact very weakly with PtdIns(3,4,5)$\_i$ in vivo and that IGF1 stimulation does not produce sufficient amounts of PtdIns(3,4,5)$\_i$ to recruit TAPP1 to the membrane.

The cellular location of PtdIns(3,4,5)$\_i$ has been studied extensively in numerous cell lines using GFP-tagged PH domains which interact specifically with PtdIns(3,4,5)$\_i$, such as GRP1 [28,33,34] or Bruton’s tyrosine kinase (BTK) [35]. These investigations have established that GFP–GRP1 and GFP–BTK PH
domains are recruited to the plasma membrane, in response to insulin, growth factors and other agonists that activate PI 3-kinases. This indicates that the bulk of cellular PtdIns(3,4,5)$P_3$ is localized at the plasma membrane. Our results validate the use of the C-terminal PH domain of TAPP1 as a probe to monitor the intracellular formation and location of PtdIns(3,4)$P_2$ selectively. In a previous study, the PH domain of PKB, which binds both PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ was used to suggest that PtdIns(3,4)$P_2$ was located at the plasma membrane in H$_2$O$_2$-stimulated cells [28]. However, in this experiment it is not possible to exclude the possibility that at least some of the membrane localization observed was caused by the interaction of PKB with PtdIns(3,4,5)$P_3$ rather than PtdIns(3,4)$P_2$ [28]. The only other protein that has been reported to interact with PtdIns(3,4)$P_2$ in vitro with some selectivity over other phosphoinositides is the PX domain of the p47$^{phox}$ subunit of the phagocytic NADPH oxidase complex [36]. However, it is unlikely that interaction of the p47$^{phox}$ PX domain with PtdIns(3,4)$P_2$ is a sufficiently specific probe for PtdIns(3,4)$P_2$ formation in vivo, because it also interacts with PtdIns(3,4,5)$P_3$, PtdIns(3,5)$P_2$ and
Our observations indicate that the bulk of PtdIns(3,4)_{P_2} generated in response to H\textsubscript{2}O\textsubscript{2} and PDGF is located at the plasma membrane. The PtdIns(3,4)_{P_2} formed in response to PDGF is likely to be generated through the dephosphorylation of PtdIns(3,4,5)_{P_2} by the 5-phosphatases SHIP1/SHIP2 [37,38]. As PtdIns(3,4,5)_{P_2} is located at the plasma membrane and the SHIP1/SHIP2 phosphatases are also recruited to the plasma membrane following stimulation of cells with growth factors [5], this would account for the plasma-membrane localization of PtdIns(3,4)_{P_2} following PDGF stimulation. In contrast, the pathway by which H\textsubscript{2}O\textsubscript{2} can increase cellular levels of PtdIns(3,4)_{P_2} is not known. However, one possibility is that H\textsubscript{2}O\textsubscript{2} activates PI 3-kinase by inhibiting tyrosine phosphatases [14], and also inhibits PTEN (phosphatase and tensin homologue deleted on chromosome 10), the major PtdIns(3,4,5)_{P_2} 3-phosphatase, but not SHIP2 (D. Bennett and C. P. Downes, unpublished work). If this were the case, we favour the view that PtdIns(3,4)_{P_2} could be generated in response to H\textsubscript{2}O\textsubscript{2} through the dephosphorylation of PtdIns(3,4,5)_{P_2}. This would be consistent with the finding that H\textsubscript{2}O\textsubscript{2} recruits TAPP1 to the plasma membrane. However, PtdIns(3,4)_{P_2} may not always be localized at the plasma membrane. As described in the Introduction, in platelets, PtdIns(3,4)_{P_2} can also be generated through the phosphorylation of PtdIns(3,4)_{P_3} by an as-yet-uncharacterized PI 4-kinase [13]. As the bulk of cellular PtdIns(3,4)_{P_3} is endosomal [39], it is possible that PtdIns(3,4)_{P_2} generated through the phosphorylation of PtdIns(3,4)_{P_3} is located at the endosomes or other intracellular compartment rather than at the plasma membrane. It will therefore be important in future studies to exploit the ability of TAPP1 or TAPP2 to bind PtdIns(3,4)_{P_2}, to investigate the location of PtdIns(3,4)_{P_2} in fibrinogen-stimulated platelets [12] or in other cells where PtdIns(3,4)_{P_2} is produced by the phosphorylation of PtdIns(3,4)_{P_3}. It should be noted that Fukui and colleagues [40] have generated a monoclonal antibody that apparently recognizes PtdIns(3,4)_{P_2} specifically and demonstrated that following H\textsubscript{2}O\textsubscript{2} stimulation of 293 cells, PtdIns(3,4)_{P_2} is localized both at the plasma membrane and at the nuclear membrane. This latter observation contrasts with our findings using TAPP1 as a probe of PtdIns(3,4)_{P_2}, in which we see no evidence of recruitment of TAPP1 to the nuclear membrane.
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