RESEARCH COMMUNICATION

Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities

Simon DOWLER*, Richard A. CURRIE†, David G. CAMPBELL*, Maria DEAK‡, Gursant KULAR†, C. Peter DOWNES† and Dario R. ALESSI*[1]

*MRC Protein Phosphorylation Unit, MSI/WTB Complex, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland, U.K., †Department of Biochemistry, MSI/WTB Complex, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland, U.K., and ‡Division of Signal Transduction Therapy, MSI/WTB Complex, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland, U.K.

The second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] is generated by the action of phosphoinositide 3-kinase (PI 3-kinase), and regulates a plethora of cellular processes. An approach for dissecting the mechanisms by which these processes are regulated is to identify proteins that interact specifically with PtdIns(3,4,5)P_3. The pleckstrin homology (PH) domain has become recognized as the specialized module used by many proteins to interact with PtdIns(3,4,5)P_3. Recent work has led to the identification of a **p**utative **p**hosphatidylinositol 3,4,5-trisphosphate-**b**inding **m**otif (PPBM) at the N-terminal regions of PH domains that interact with this lipid. We have searched expressed sequence tag databases for novel proteins containing PH domains possessing a PPBM. Surprisingly, many of the PH domains that we identified do not bind PtdIns(3,4,5)P_3, but instead possess unexpected and novel phosphoinositide-binding specificities *in vitro*. These include proteins possessing PH domains that interact specifically with PtdIns(3,4)P_2 [TAPP1 (**ta**ndem **P**H-domain-containing **p**rotein-1) and TAPP2], PtdIns4*P* [FAPP1 (phosphatidylinositol-**f**our-phosphate **a**da**p**tor **p**rotein-1)], PtdIns3*P* [PEPP1 (**p**hosphatidylinositol-thr**ee**-**p**hosphate-binding **P**H-domain protein-**1**) and AtPH1] and PtdIns(3,5)P_2 (centaurin-β2). We have also identified two related homologues of PEPP1, termed PEPP2 and PEPP3, that may also interact with PtdIns3*P*. This study lays the foundation for future work to establish the phospholipid-binding specificities of these proteins *in vivo*, and their physiological role(s).

Key words: AtPH1, centaurin-β2, phosphoinositide 3-kinase, PtsIns3*P*-binding PH domain protein-1 (PEPP1), PtsIns4*P* adaptor protein-1 (FAPP1), tandem pleckstrin-homology (PH)-domain-containing **p**rotein-1 (TAPP1), TAPP2.

INTRODUCTION

Stimulation of cells with growth factors and insulin activates members of the phosphoinositide 3-kinase (PI 3-kinase) family, which phosphorylate phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] at the D-3 position of the inositol ring to generate the lipid second messenger, PtdIns(3,4,5)P_3 [1]. A group of proteins has been identified that possess a certain type of pleckstrin homology (PH) domain that interacts specifically with PtdIns(3,4,5)P_3 and, often, its immediate breakdown product, PtdIns(3,4)P_2, also thought to be a signalling lipid (reviewed in [2]). These include the serine/threonine-specific protein kinases, protein kinase B (PKB) and 3-phosphoinositide-dependent protein kinase-1 (PDK1) [3], Bruton's tyrosine kinase (BTK) [4], the adaptor proteins DAPP1 (**d**ual **a**daptor for **p**hosphotyrosine and 3-**p**hosphoinositides) [5,6] and Gab1 [7], as well as the ADP-ribosylation factor (ARF) GTPase-activating protein (GAP) centaurin-α [8] and the ARF guanine-nucleotide-exchange factor, Grp1 [9,10].

The molecular basis by which certain PH domains are able to interact with PtdIns(3,4,5)P_3 has not been established definitively. However, recent work indicates that six conserved residues that lie in the N-terminal region of the PH domain in a Lys-Xaa-Sma-Xaa$_{6-11}$-Arg/Lys-Xaa-Arg-Hyd-Hyd motif (where 'Xaa' is any amino acid, 'Sma' is a small amino acid and 'Hyd' is a hydrophobic amino acid) appear to correlate with high-affinity binding of PtdIns(3,4,5)P_3 [11]. To date, all of the specific PtdIns(3,4,5)P_3-binding proteins identified possess this **p**utative **P**tdIns(3,4,5)P_3 **b**inding **m**otif (PPBM) (see Table 1). Mutation of certain of the conserved residues in the PPBM in some PH domains has been shown to abolish interaction with PtdIns(3,4,5)P_3 [11]. Recent structural studies of the PH domain of BTK bound to the head group of PtdIns(3,4,5)P_3 indicate that the basic amino acids in the PPBM may form direct interactions with the monoester phosphate groups of PtdIns(3,4,5)P_3 [2,12,13].

To identify novel proteins that interact with PtdIns(3,4,5)P_3, we searched expressed sequence tag (EST) databases for proteins possessing a PH domain containing a PPBM. Surprisingly,

Abbreviations used: PI 3-kinase, phosphoinositide 3-kinase; PH, pleckstrin homology; PKB, protein kinase B; PDK1, 3-phosphoinositide-dependent protein kinase-1; BTK, Bruton's tyrosine kinase; DAPP1, dual adaptor for phosphotyrosine and 3-phosphoinositides; ARF, ADP-ribosylation factor; GAP, GTPase-activating protein; PPBM, **p**utative **p**hosphatidylinositol 3,4,5-trisphosphate-**b**inding **m**otif; EST, expressed sequence tag; GST, glutathione S-transferase; TAPP, **ta**ndem **P**H-domain-containing **p**rotein; FAPP1, phosphatidylinositol-**f**our-phosphate **a**da**p**tor **p**rotein-1; PEPP1, **p**hosphatidylinositol-thr**ee**-**p**hosphate-binding **P**H-domain protein-**1**; HEK-293, human embryonic kidney-293; NCBI, National Center for Biotechnology Information; PDZ, postsynaptic density protein (**P**SD-95)/*Drosophila* disc large-tumour suppressor (**D**lg)/tight junction protein (**Z**O1).

[1] To whom correspondence should be addressed (e-mail s.j.dowler@dundee.ac.uk).

The nucleotide sequence data reported will appear in the National Center for Biotechnology Information database under the accession nos. as shown: human TAPP1 (full-length sequence), AF286160; human TAPP2 (partial sequence), AF286164; mouse TAPP2 (full-length sequence), AF286161; human FAPP1 (full-length sequence), AF286162; mouse FAPP1 (full-length sequence), AF286163; human PEPP1 (full-length sequence), AY007233.

instead of interacting with PtdIns(3,4,5)P_3, six of the expressed PH domains bound specifically to other phosphoinositides.

EXPERIMENTAL

Materials

Unless otherwise indicated, all phosphoinositides used in this study were dipalmitoyl derivatives obtained from Cell Signals (Lexington, KY, U.S.A.), which were analysed by TLC and were found to migrate as single products. *sn*-1-Stearoyl-2-arachidonoyl-D-PtdIns(3,4,5)P_3 [14] was a gift from Piers Gaffney (Ludwig Institute for Cancer Research, University College London, London, U.K.). Hybond-C extra was from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.), Hi-Fidelity PCR kits were from Roche Diagnostics (Welwyn Garden City, Herts., U.K.), human tissue (Catalogue no. 7780-1), mouse tissue (Catalogue no. 7762-1) and human cancer cell-line (Catalogue no. 7757-1) multiple-tissue Northern blots were from Clontech Laboratories (UK Limited, Basingstoke, U.K.), Human Universal cDNA Library was from Stratagene (Cambridge, U.K.); pCR 2.1Topo vector and pre-cast SDS/polyacrylamide gels were from Invitrogen (Carlsbad, CA, U.S.A.). DAPP1 [5] and Grp1 [9] were expressed as fusion proteins with glutathione S-transferase (GST), and the PH domain of human phospholipase Cδ1 (residues 20–184) fused to GST was expressed in *Escherichia coli*.

General methods

Restriction-enzyme digestions, DNA ligations, site-directed mutagenesis and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing.

Cloning of PH domains and preparation of expression constructs

All the human and mouse ESTs were obtained from the I.M.A.G.E. Consortium [15] and sequenced. The plant EST (accession no. T04439) encoding a full-length clone of AtPH1 was obtained from the *Arabidopsis* Biological Research Centre (Ohio University, OH, U.S.A.). The sequence of each EST was verified and the full-length PH domain of each EST was amplified by PCR using the Hi-Fidelity PCR system with primers designed to incorporate a Kozak site, an initiator ATG codon followed by a Myc epitope tag, and a stop codon after the PH domain. The region of each protein that was amplified using the indicated EST as template was as follows: human TAPP1 (residues 95–404; accession no. AI216176), mouse TAPP2 (residues 174–425; accession no. AA111410), human FAPP1 (residues 1–99; accession no. W32183), *Arabidopsis thaliana* AtPH1 (full-length protein, residues 1–145; accession no. T04439), human PEPP1 (residues 15–169; accession no. N31123), mouse centaurin-β2 (residues 266–390; accession no. AA967911), putative human homologue of rat LL5α (sequence Ser-Glu-Ser-Ala to Gln-Phe-Met-Asn; accession no. AA863428), putative human isoform of LL5α, which we have termed LL5β (sequence Arg-Lys-Glu-Asp to His-Phe-Leu-Leu; accession no. AA461369), mouse pleckstrin-2 (residues 1–249; accession no. AI326844), human evectin-2 (residues 1–167; accession no. AA101447) and human PH30 (sequence Asn-Ser-Ser-Ile to Ile-Ser-Asp-Ala; accession no. AI827615). The PCR products were resolved on 1% (w/v) agarose gels, gel-purified, cloned into the pCR2.1 TOPO vector, sequenced and subcloned into either the *E. coli* pGEX-4T-1 expression vector or the mammalian pEBG2T vector, which codes for the expression of these proteins with a GST tag at the N-terminus.

Expression of GST–PH domains in *E. coli*

The pGEX-4T-1 constructs encoding the PH domains of FAPP1, AtPH1, PEPP1, LL5α, LL5β, evectin-2 and PH30 were transformed into BL21 *E. coli* cells and a 500 ml culture was grown at 37 °C in Luria broth containing 100 μg/ml ampicillin until the attenuance at 600 nm was 0.6. Isopropyl β-D-thiogalactoside (250 μM) was added, and the cells were cultured for a further 16 h at 26 °C. The cells were resuspended in 25 ml of ice-cold Buffer A [50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (v/v) 2-mercaptoethanol and 'complete' proteinase inhibitor cocktail (one tablet per 25 ml; Roche Diagnostics)], lysed by one round of freeze–thawing, and the lysates were sonicated to fragment the DNA. The lysates were centrifuged at 20000 ***g*** for 30 min at 4 °C, and the supernatant was then filtered through a 0.44 μm filter and incubated for 60 min on a rotating platform with 1 ml of glutathione–Sepharose pre-equilibrated in Buffer A. The suspension was centrifuged for 1 min at 3000 ***g***, the beads were washed three times with 15 ml of Buffer A containing 0.5 M NaCl, and then a further ten times with 15 ml of Buffer B (50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 10 mM 2-mercaptoethanol and 0.27 M sucrose). The protein was eluted from the resin at ambient temperature by incubation with 2 ml of Buffer B containing 20 mM glutathione, and the beads were removed by filtration through a 0.44 μm filter. The eluate was divided into aliquots, snap-frozen in liquid nitrogen and stored at −80 °C.

Expression of GST–PH domains in human embryonic kidney-293 (HEK-293) cells

As the PH domains of TAPP1, TAPP2, centaurin-β2 and pleckstrin-2 were significantly degraded when expressed in bacteria (results not shown), these were expressed as GST-fusion proteins in HEK-293 cells. For the expression of each construct, twenty 10-cm-diameter dishes of HEK-293 cells were cultured, and each dish was transfected with 5 μg of the pEBG-2T construct using a modified calcium phosphate method [16]. Post-transfection (36 h), the cells were lysed in 1 ml of ice-cold Buffer A, the lysates were pooled, centrifuged at 4 °C for 10 min at 13000 ***g***, and the GST-fusion proteins were purified by affinity chromatography on glutathione–Sepharose before storage, as described above.

Cloning of TAPP1, TAPP2 and FAPP1

Full-length human TAPP1, full-length mouse TAPP2, partial mouse TAPP1, partial human TAPP2 and full-length human and mouse FAPP1 sequences were deduced by sequencing the EST clones listed in Table 3. Several EST clones possessed identical sequences, the same in-frame stop codon 5′ to the predicted initiating ATG codon, and a stop codon at the same position at the 3′-end of the gene. The constructs used to express full-length and deletion mutants of TAPP1 and TAPP2 were generated by PCR, using as a template ESTs encoding full-length human TAPP1 (accession no. AI216176) and full-length mouse TAPP2 (accession no. AA111410). The PCR primers used were designed to incorporate a Kozak site and an initiating ATG codon followed by a FLAG epitope tag, and the resulting PCR product was subcloned into the pEBG2T mammalian expression

vector. The sequences of primers used in the present study are available, on request, from the authors.

Cloning of PEPP1, PEPP2 and PEPP3

The partial sequence of human PEPP1 that contains the 5′-end of the coding sequence was obtained by sequencing of ESTs with National Center for Biotechnology Information (NCBI) accession nos. N49341 and N31123. To obtain a full-length cDNA encoding PEPP1, we screened a Stratagene Human Universal cDNA Library with a DNA probe corresponding to the N-terminal 15–169 residues of PEPP1, and isolated a full-length PEPP1 cDNA that had a stop codon 5′ to the predicted initiating ATG codon and an open reading frame encoding 779 amino acids followed by a stop codon. Interrogation of the EST databases with the full-length PEPP1 sequence identified two closely related isoforms of this protein, termed PEPP2 and PEPP3. The sequence of human PEPP2 was deduced by sequencing the EST clones A1808805 (kidney), AA232124 (brain), W91917 (fetal liver and spleen) and AI638629 (germ-cell line). The sequence of PEPP2 is likely to be full length because there is a stop codon 5′ to the predicted initiating ATG codon. The sequence of human PEPP3 (also termed KIAA0969) has been deposited on the database as part of a project cloning large cDNAs expressed in brain [17]. There are also three ESTs available encoding PEPP3 that have accession nos. AI739438, BE303674 and F23241.

Northern blot analysis

cDNA corresponding to full-length human TAPP1, partial human TAPP2 (residues 18–304), partial human PEPP1 (residues 15–169), partial human PEPP2 (residues 154–654) and mouse partial mouse centaurin-β2 (residues 266–390) were labelled with [^{32}P]α-dATP by random priming using a multiprime DNA labelling kit (Amersham Pharmacia Biotech). These probes were then used to screen Northern blots using Rapid-Hyb Buffer (Amersham Pharmacia Biotech), according to the manufacturer's protocol.

Protein–lipid overlay assay

To assess the phosphoinositide-binding properties of each PH domain, a protein–lipid overlay assay was performed using the GST-fusion proteins, as described previously [5,18]. Briefly, 1 μl of lipid solution containing 1–100 pmol of phospholipids dissolved in a mixture of choroform/methanol/water (1:2:0.8, by vol.) was spotted on to Hybond-C extra membrane and allowed to dry at room temperature for 1 h. The membrane was blocked in 3% (w/v) fatty acid-free BSA in TBST [50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween 20] for 1 h. The membrane was then incubated overnight at 4 °C with gentle stirring in the same solution containing 0.2 μg/ml of the indicated GST-fusion protein. The membranes were washed six times over 30 min in TBST, and then incubated for 1 h with 1:1000 dilution of anti-GST monoclonal antibody (Sigma, St Louis, MO, U.S.A.). The membranes were washed as before, then incubated for 1 h with 1:5000 dilution of anti-mouse–horseradish peroxidase conjugate (Pierce, Rockford, IL, U.S.A.). Finally, the membranes were washed 12 times over 1 h in TBST, and the GST-fusion protein that was bound to the membrane by virtue of its interaction with phospholipid was detected by enhanced chemiluminescence.

Table 1 Alignment of proteins possessing a putative PtdIns(3,4,5)P_3-binding motif

Alignment of the amino acid residues of the N-terminal region of PH domains containing at least five out of the six conserved residues in the motif Lys-Xaa-Sma-Xaa$_{6–11}$-Arg/Lys-Xaa-Arg-Hyd-Hyd, where 'Xaa' is any amino acid, 'Sma' is a small uncharged amino acid and 'Hyd' is a hydrophobic amino acid. Amino acids are shown by the single-letter code, and asterisks indicate accession nos. of the EST used to amplify, by PCR, the PH domain for which lipid-binding properties are illustrated in Figure 2. Org., organization.

(a)

Known PtdIns(3,4,5)P_3-binding proteins	Protein	Accession no.	Species	Protein function	Ref.
KEGWLHKRGEYIKY-----WRPRYFLLKND	PKB	S33364	Mouse	Ser/Thr kinase	[2]
ENNLILKMGPVDKRKGLF-ARRRQLLTEGP	PDK1	AAC51825	Human	Ser/Thr kinase	[2]
LESIFLKRSQQKKKTSPLNFKKRLFLLTVH	BTK	Q06187	Human	Tyrosine kinase	[3]
KEGYLTKQGGLVKT-----WKTRWFTLHRN	DAPP1	AF163254	Human	Unknown (adaptor?)	[4]
CSGWLRKSPPEKKLKRYA-WKRRWFVLRSG	Gab1	AAC50380	Human	Docking protein	[6]
KEGYMEKTGPKQTEG----FRKRWFTMDDR	Centaurin-α1	JC7091	Human	ARF GAP	[7]
REGWLLKLGGRVKT-----WKRRWFILTDN	GRP1	AF001871	Mouse	ARF GEF	[8]

(b)

PH domains characterized in this study	Protein	Accession no.	Species	Protein function	Ref.
KAGYCVKQGAVMKN-----WKRRYFQLDEN	TAPP1	AI216176*	Human	Unknown (adaptor?)	
KSGYCVKQGNVRKS-----WKRRFFALDDF	TAPP2	AA111410*	Mouse	Unknown (adaptor?)	
MEGVLYKWTNYLTG-----WQPRWFVLDNG	FAPP1	W32183*	Human	Unknown (adaptor?)	
IRGWLHKQDSSGLRL----WKRRWFVLSGH	PEPP1	N31123*	Human	Unknown	
RSGWLTKQGDYIKT-----WRRRWFVLKRG	AtPH1	T04439*	*Arabidopsis*	Unknown (adaptor?)	[24]
MEGYLFKRASNAFKT----WNRRWFSIQNS	Centaurin-β2	AA967911*	Mouse	ARF GAP	
KSGWLLRQSTILKR-----WKKNWFDLWSD	Evectin-2	AA101447*	Human	Golgi trafficking?	[21]
CRGYLVKMGGKIKS-----WKKRWFVFDRL	LL5α	AA863428*	Human	Unknown	[17]
CRGFLIKMGGKIKT-----WKKRWFVFDRN	LL5β	AA461369*	Human	Unknown	
KEGFLVKRGHIVHN-----WKARWFILRQN	Pleckstrin-2	AI385784*	Mouse	Cytoskeletal Org.?	[18]
FEGTLYKRGALLKG-----WKPRWFVLNVT	PH30	AI827615*	Human	Nuclear phosphatase?	[20]

BIACore measurements of PH domain-lipid interactions

Kinetic analyses of the interactions between the GST–PH domain fusions and the polyphosphoinositides were made using surface-plasmon-resonance-based procedures, as described previously [5,19], with the following modifications. The mole-percentage of the test polyphosphoinositide was reduced from 1 % to 0.1 %. This helped to minimize any mass-transport limitation in the binding interaction, and increased the rate of lipid immobilization on the chip. The intracellular buffer was supplemented with 0.27 M sucrose to reduce the bulk refractive-index changes associated with the addition of Buffer B. Proteins were injected over the monolayers at concentrations ranging from 1 μM to 10 nM. Data were analysed using the bimolecular-interaction model and the global-fitting feature of the BIAevaluation 3 software for several sensorgrams at different protein concentrations. GST–PH domain binding to phosphoinositides does not fit well to this model, because of the slow dissociation of the protein from the surface [5,19]. Therefore, instead of calculating the apparent K_d values for the interaction of the GST–PH domain proteins with phosphoinositides, the relative binding affinities of each protein relative to the binding of full-length GST–TAPP1 with PtdIns(3,4)$P_2$was determined instead.

RESULTS

Identification of novel or uncharacterized PH domains

The NCBI/European Molecular Biology Laboratory (EMBL)/Protein Data Bank EST databases were interrogated with the amino acid sequences encoding the PH domains of human PKBα, PDK1, Grp1 and DAPP1. These searches revealed 11 partial sequences encoding either novel or previously uncharacterized PH-domain-containing proteins possessing at least five of the six conserved residues in the PPBM (Table 1). We cloned the entire PH domains of each of these proteins (see the Experimental section), which are described in Table 1. They were expressed in *E. coli* or HEK-293 cells as fusions to GST, and purified by affinity chromatography on glutathione–Sepharose. Homogeneous Coomassie-Blue-stained bands were observed for each product, and these proteins migrated with the expected molecular masses on SDS/PAGE (Figure 1).

We studied the specificity and affinity of interaction of the PH domains for phosphoinositide lipids using either a protein–lipid overlay assay [5] (Figure 2), or the more quantitative surface-plasmon-resonance-based approach [19] (Table 2). For the protein–lipid overlay assay, serial dilutions of phosphoinositides were spotted on to a nitrocellulose membrane and incubated with the indicated GST–PH domain fusion protein or GST–DAPP1 [which binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2] [5], GST–Grp1 [which binds PtdIns(3,4,5)P_3 only] [9] and GST–phospholipase Cδ1 [which binds PtdIns(4,5)P_2 only] [20] as controls. The membranes were then washed and immunoblotted with a GST antibody to detect GST-fusion proteins that were bound to the membrane by virtue of their interaction with lipid. For the surface-plasmon-resonance-based assay, the relative affinities of the GST–PH domain fusion proteins resulting from their interaction with a supported lipid monolayer containing a low mole fraction of phosphoinositide was determined (Table 2). Both these assays yielded comparable results for the lipid-binding specificities and relative affinities of the PH domains that we have isolated. As discussed below, six of the PH domains that we identified did not bind to *sn*-1,2-dipalmitoyl-D-PtdIns(3,4,5)P_3 (see Figure 6) or *sn*-1-stearoyl-2-arachidonoyl-D-PtdIns(3,4,5)P_3 (results not shown), but did interact with other phosphoinositides with various affinities and specificities. In contrast, the PH domains derived from the proteins LL5α [21], a closely related isoform to LL5α not described previously that we have termed LL5β, pleckstrin-2 [22,23] and a protein that we

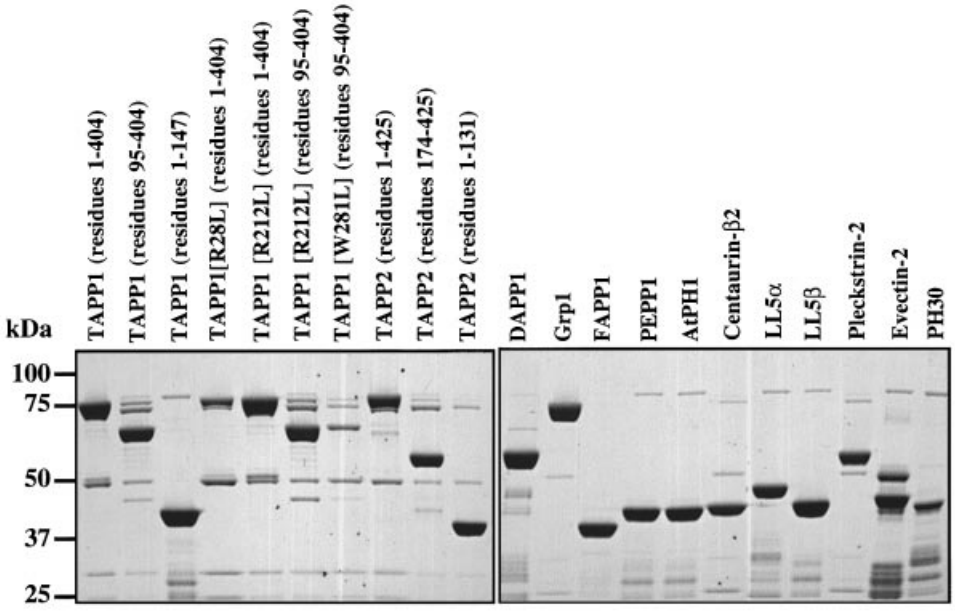


Figure 1 SDS/polyacrylamide gel of purified GST–PH domains

Of the indicated purified GST–PH domain fusions [except for the TAPP1 Trp-281 → Leu (W281L) mutant (0.5 μg), which expressed poorly], 2 μg was electrophoresed on SDS/polyacrylamide gels (4–12% gradient) and stained with Coomassie Blue. The positions of the molecular-mass markers (Bio-Rad Precision Markers) are indicated. TAPP1, TAPP2, centaurin-β2 and pleckstrin-2 constructs were expressed in HEK-293 cells and FAPP1, PEPP1, AtPH1, LL5α, LL5β, evectin-2 and PH30 were expressed in *E. coli*.

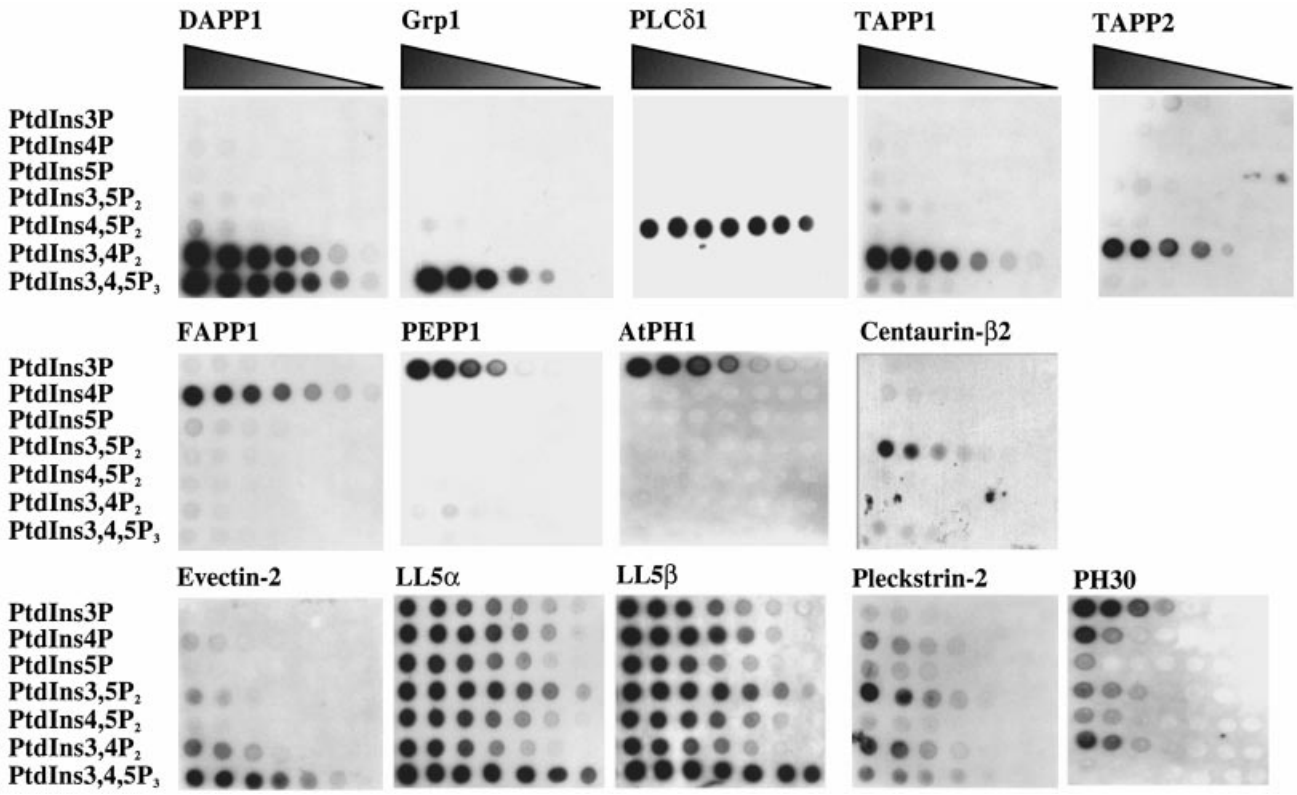


Figure 2 Phosphoinositide binding properties of the novel PH domains

The ability of the indicated GST-fusion proteins to bind a variety of phosphoinositides was analysed using a protein–lipid overlay assay. Serial dilutions of the indicated phosphoinositides (100 pmol, 50 pmol, 25 pmol, 12.5 pmol, 6.3 pmol, 3.1 pmol and 1.6 pmol) were spotted on to nitrocellulose membranes, which were then incubated with the purified GST-fusion proteins. The membranes were washed, and the GST-fusion proteins bound to the membrane by virtue of their interaction with lipid were detected using a GST antibody. A representative of at least three separate experiments is shown.

Table 2 Relative affinities of PEPP1, FAPP1 wild-type and mutant TAPP1 and TAPP2 for binding to phosphoinositides as measured by surface plasmon resonance

The binding of the indicated GST-fusion protein phosphoinositides incorporated into supported phosphatidylcholine monolayers was measured as described in the Experimental section. The apparent affinities were determined by global fitting of the association and dissociation curves to a 1:1 binding model, and were used to rank the binding affinity relative to that of TAPP1 to PtdIns(3,4)P_2, which was approx. 5 nM. The numeric relative affinity binding data are indicated in bold. FL, full-length protein; NT-PH, N-terminal PH domain; CT-PH, C-terminal PH domain; NB, no binding detected; ND, not determined; R212L, a mutant bearing an Arg-212 → Leu substitution; R28L, a mutant bearing an Arg-28 → Leu substitution.

Phosphoinositide	DAPP1	PDK1	PEPP1	FAPP1	FL-TAPP1	FL-TAPP2	FL-TAPP1 (R212L)	FL-TAPP1 (R28L)	CT-PH TAPP1	CT-PH TAPP1 (R212L)	NT-PH TAPP1
PtdIns3*P*	NB	NB	**65**	NB	NB	NB	ND	ND	ND	ND	ND
PtdIns4*P*	NB	NB	NB	**4**	NB	NB	ND	ND	ND	ND	ND
PtdIns5*P*	NB	NB	NB	NB	NB	NB	ND	ND	ND	ND	ND
PtdIns(3,4)P_2	ND	ND	NB	NB	**1**	**5**	NB	**5.6**	**5.4**	**19.6**	NB
PtdIns(3,4,5)P_3	**0.6**	**12**	NB	NB	NB	NB	**ND**	ND	ND	ND	ND
PtdIns(3,5)P_2	NB	NB	NB	NB	NB	NB	ND	ND	ND	ND	ND
PtdIns(4,5)P_2	NB	NB	NB	NB	NB	NB	ND	ND	ND	ND	ND

have called PH30, which displays 70% identity with the nuclear dual-specificity phosphatase [24] (accession no. AAC39675), interacted with several phosphoinositides (Figure 2). The PH domain of a protein of unknown function, termed evectin-2, which localizes to post-Golgi membranes [25], showed a moderate affinity for PtdIns(3,4,5)P_3, but also interacted more weakly with several other phosphoinositides (Figure 2). None of the PH domains for which lipid-binding properties were investigated (as shown in Figure 2) interacted with phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol in the protein–lipid overlay assay (results not shown).

TAPP1 and TAPP2 bind specifically to PtdIns(3,4)P_2

Two of the novel sequences identified encoded related proteins, which were termed TAPP1 and TAPP2 (Table 1). Clones encoding the full-length human TAPP1 (accession no. AF-286160), mouse TAPP2 (accession no. AF286161) and human

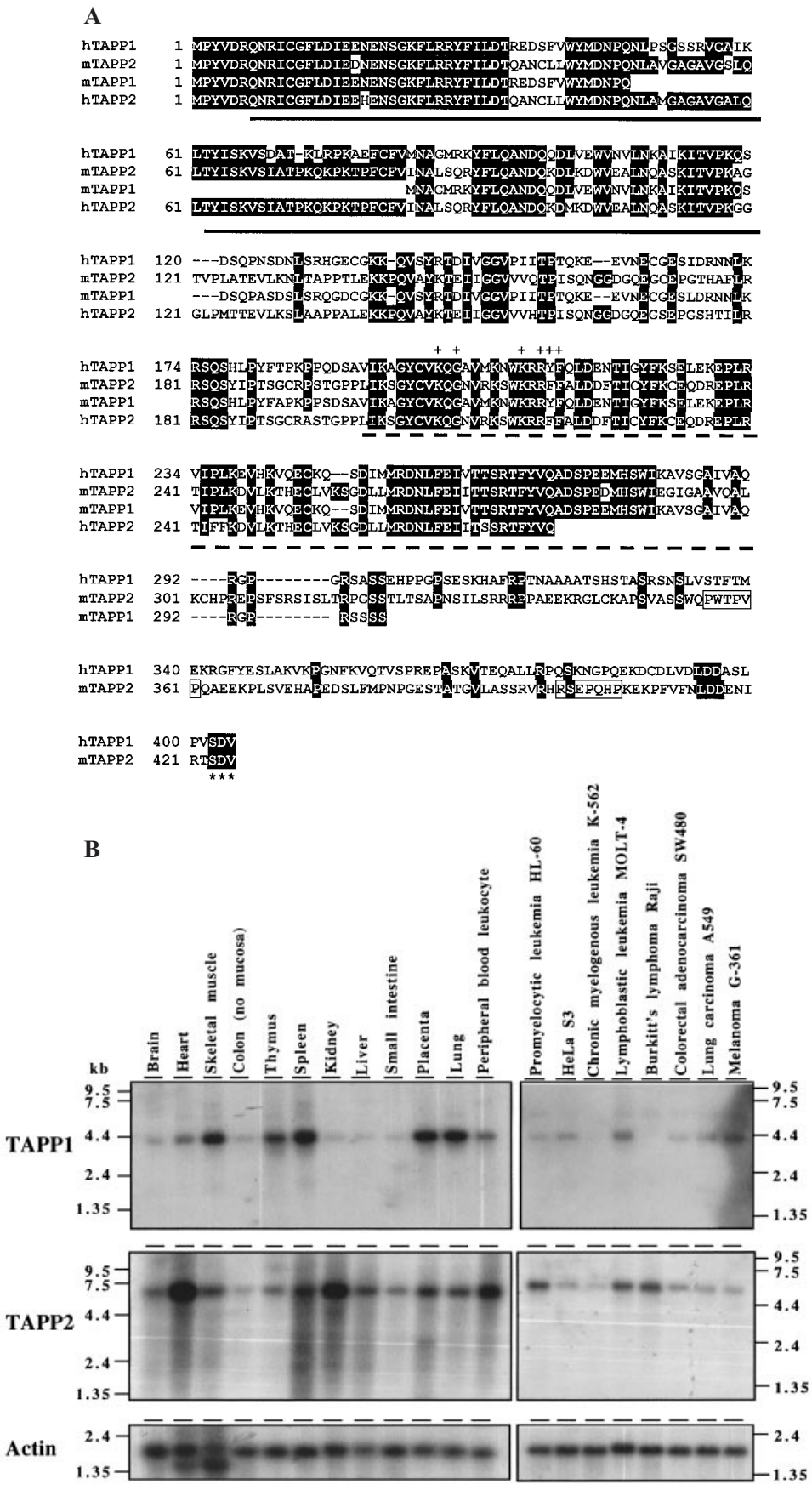


Figure 3 For legend, see opposite page

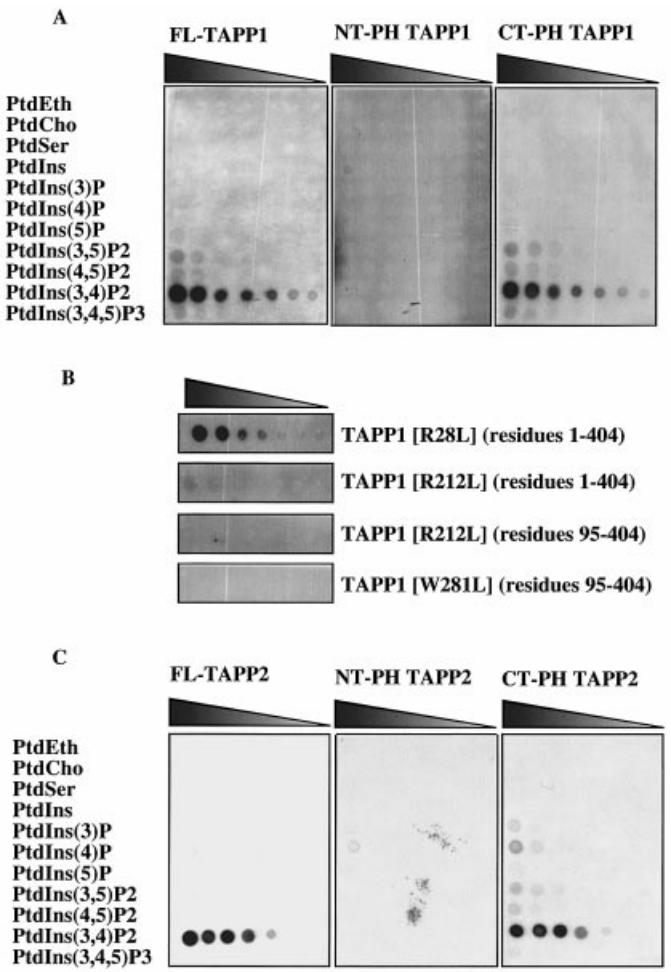


Figure 4 Comparison of the phosphoinositide-binding properties of the N-terminal and C-terminal PH domains of TAPP1 and TAPP2

The ability of wild-type and mutant forms of full-length (FL) and isolated N-terminal (NT) and C-terminal (CT) PH domains of TAPP1 and TAPP2 GST-fusion proteins to interact with phosphoinositides were analysed using a protein–lipid overlay assay. Serial dilutions of the indicated phosphoinositides (100 pmol, 50 pmol, 25 pmol, 12.5 pmol, 6.3 pmol, 3.1 pmol and 1.6 pmol) were spotted on to a nitrocellulose membrane, which was then incubated with the indicated purified GST-fusion proteins. The membranes were washed and the GST-fusion proteins bound to the membrane by virtue of their interactions with lipid were detected using a GST antibody. A representative experiment of three is shown. The isolated N-terminal PH domain of human TAPP1 comprises residues 1–147, the isolated C-terminal PH domain of human TAPP1 comprises residues 95–404, the isolated N-terminal PH domain of mouse TAPP2 comprises residues 1–131 and the isolated C-terminal PH of mouse TAPP2 comprises residues 174–425. PtdEth, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine.

TAPP2 (accession no. AF286164) were isolated as described in the Experimental section. Human TAPP1 is a protein of 404 amino acids, and mouse TAPP2 is a protein of 425 amino acids (Figure 3A). A stop codon immediately 5′ to the predicted initiating ATG codon indicates that both human and mouse TAPP1 and TAPP2 protein sequences are full length. Analysis of the TAPP1 and TAPP2 sequences revealed the presence in each protein of two PH domains, of which only the C-terminal PH domain possesses the PPBM (Figure 3A). The amino acid sequences of TAPP1 and TAPP2 are 58 % identical over the first 300 amino acids, which encompasses both of the PH domains. There is little homology between the C-terminal 100 residues of TAPP1 and TAPP2, except that seven out of the eleven C-terminal amino acids of TAPP1 and TAPP2 are identical. The last three residues of TAPP1 and TAPP2 conform to the minimal sequence motif (Ser/Thr-Xaa-Val/Ile) [26,27] required for

Figure 3 Amino acid sequence and tissue distribution of TAPP1 and TAPP2

(**A**) The alignment of the human and mouse TAPP1 and TAPP2 sequences is shown. The identities are shown in reversed-out white lettering on a black background. The DNA sequences encoding the human (h) and mouse (m) TAPP1 shown are available from the NCBI database (accession nos. for hTAPP1, hTAPP2 and mTAPP2 are AF286160, AF286164 and AF286161 respectively). The amino acid residues corresponding to the N-terminal and C-terminal PH domains are indicated by a continuous black line and a broken line respectively. The residues that comprise the putative SH3-domain-binding proline-rich motif of TAPP2 are boxed. The residues of the C-terminal PH domain of TAPP1 and TAPP2 that make up the PPBM are indicated by ' + '. The C-terminal Ser-Asp-Val sequence of TAPP1 and TAPP2 that could interact with proteins possessing a PDZ domain(s) is shown by asterisks. The sequences of mTAPP1 and hTAPP2 are not fully characterized, and the residues that are not known are indicated by blank spaces. (**B**) TAPP1 and TAPP2 cDNAs were labelled with [^{32}P]α-dATP using random primers (see the Experimental section), and subsequently used to probe a Northern blot containing polyadenylated RNA isolated from the indicated human tissues and cancer cell lines. The blot was washed and autoradiographed. The TAPP1 and TAPP2 probes were observed to hybridize to a 4 kb and a 6 kb message respectively.

Table 3 Tissue origin of ESTs encoding TAPP1, TAPP2, PEPP1 and FAPP1

ESTs that we have sequenced also have their I.M.A.G.E. Consortium Clone IDs featured in parentheses.

Protein	Species	Tissue	NCBI Accession (I.M.A.G.E. Clone ID)
TAPP1	Human	Parathyroid tumour	W56032, W63712 (326517)
		Fetal heart	AA054961
		Lung	AI191308, AI216176 (1884429)
		Colon	AI709038
		Kidney	AA875839, AI343801
		Skeletal muscle	AA211648
		Melanocyte	N31136
		Testis	AI343801
		Olfactory epithelium	AL046495
		Germinal centre B cell	AA740729 (1286305)
		Fetal liver	H78048, H90955
		Uterus	AA150283 (491669)
		Placenta	R62858
		Testis	AA429617
		Fetal liver	R91752
	Mouse	Thymus	AA762924
		Kidney	AI987596 (2158944)
		Embryo	AA388896 (569145)
	Zebrafish	Pooled	AI497344, AI878142
		Fin regenerates	AW595189
TAPP2	Human	Germinal centre B cells	AA721234 (1300983)
		Fetal lung	AI185428 (1742690)
		Pooled tumours	AA975814 (1589519)
		Brain	AA985353, AW408638
	Mouse	Embryo	AA111410 (557355)
		Thymus	AA118260, AI447504 (574391)
		Myotubes	AI592480, AI591454 (1162924)
	Chicken	Bursa of Fabricius	AJ393764, AJ395418, AJ393899
FAPP1	Human	Multiple sclerosis	N79274 (287618)
		Germinal centre B cells	AA481205 (815143), AA481224 (815169), AI221252 (1842552)
		Bowel	BE136879
		Testis	AA431220
		Lung carcinoid	AW340998, AW341035
		Fetal heart	W73345
		Colon tumour	AI337400
		Pancreatic islet	W52895 (338749)
		Aorta endothelial	AA301959
		Germ cell tumour	AI341371
		Pooled	AI246428, AI242688, AA453702 (813820), AA724575 (1327281)
		Parathyroid tumour	W32183 (321321)
		Uterus	AI161122 (1721404)
		Total fetus	AA463817 (796517)
	Mouse	Embryo	AA681116 (1134498)
		Macrophages	AA867335 (1293870)
		Tumour	AW412246 (2812588)
		Spleen	AA184412
		Total fetus	AA048334 (477463)
		Heart	AA419963 (847595)
	Rat	Ovary	AI177017
		Pooled	AI071963
	Xenopus	Unfertilized egg	AW644282
	Zebrafish	Pooled	AW174299
PEPP1	Human	Melanocytes	N49341 (272085), N31123 (265349)
		Melanoma	AL135424, AL135565

binding to a PDZ domain ['PDZ' is an acronym derived from postsynaptic density protein (**P**SD-95)/*Drosophila* disc large-tumour suppressor (**D**lg)/tight junction protein (**Z**O1)]. Apart from two proline-rich regions towards the C-terminus of TAPP2, which could form a binding site for an SH3 domain (Figure 3), no other known catalytic domains are present. Interrogation of the NCBI human genome database with the TAPP1 sequence indicated that it is located on chromosome 10q25.3–q26.2. Although the genomic fragment that encompasses TAPP2 (accession no. AC067817) has been sequenced, its chromosomal location is not yet known.

The isolated C-terminal PH domains of TAPP1 and TAPP2 (which possess the PPBM), when expressed as GST-fusion proteins, interacted with PtdIns(3,4)P_2, but did not bind to PtdIns(3,4,5)P_3 or any other phosphoinositides tested (Figure 2). Surface-plasmon-resonance studies also indicated that the isolated C-terminal PH domain of TAPP1 and TAPP2 interacted with high specificity with PtdIns(3,4)P_2 (Table 2). TAPP1 possesses a 5-fold higher affinity for PtdIns(3,4)P_2 than TAPP2. The affinity of TAPP1 for PtdIns(3,4)P_2 is similar to that at which DAPP1 binds PtdIns(3,4,5)P_3, and 10-fold higher than the affinity with which PDK1 interacts with PtdIns(3,4,5)P_3 (Table 2). The N-terminal PH domain of TAPP1 and TAPP2 failed to interact with any phosphoinositide tested (Figure 4A and Table 2). The full-length GST–TAPP1 (Figure 4A and Table 2) and full-length GST–TAPP2 (Figure 4C and Table 2) fusion proteins interacted specifically with PtdIns(3,4)P_2. Mutation of the conserved Arg-212 to leucine in the PPBM of the C-terminal PH domain of TAPP1 abolished or greatly reduced the interaction of both full-length TAPP1 and the isolated C-terminal PH domain with PtdIns(3,4)P_2 (Figure 4B and Table 2). The mutation of the residue Arg-28 to leucine in the N-terminal PH domain of TAPP1, which lies in a position equivalent to that of Arg-212 in the C-terminal PH domain, did not affect the interaction of full-length GST–TAPP1 with PtdIns(3,4)P_2 as observed in the qualitative protein–lipid overlay assay (Figure 4B), but a 5-fold decrease in the affinity of this mutant for PtdIns(3,4)P_2 was observed in the more quantitative surface-plasmon-resonance binding assay (Table 2). Consistent with a role for the N-terminal PH domain of TAPP1 in contributing towards the affinity with which the C-terminal PH domain binds to PtdIns(3,4)P_2, the isolated C-terminal PH domain of TAPP1 bound to PtdIns(3,4)P_2 with a 5-fold lower affinity than full-length TAPP1 (Table 2). As expected, the mutation to leucine of the conserved tryptophan residue (Trp-281) found in all PH domains abolished the interaction of the isolated C-terminal PH domain of TAPP1 with PtdIns(3,4)P_2 (Figure 4B).

The tissue distribution of TAPP1 and TAPP2 mRNA was investigated by Northern blot analysis. TAPP1 was detected as a 4 kb transcript in all tissues examined, with the highest levels observed in skeletal muscle, spleen, lung, thymus and placenta (Figure 3B). TAPP2 was detected as a 6 kb transcript in all tissues examined, with the highest levels observed in heart and kidney (Figure 3B). We identified many ESTs encoding TAPP1 and TAPP2 in the databases derived from several tissues (Table 3), indicating that TAPP1 and TAPP2 are widely expressed proteins.

FAPP1 is a specific PtdIns4*P*-binding protein

The PH domain containing the protein termed FAPP1 (Table 1), possessing a glutamine instead of lysine or arginine at the third conserved residue of the PPBM, exhibited a high affinity for PtdIns4*P*, but did not bind to any other phosphoinositide (Figure 2 and Table 2). The full-length human and mouse FAPP1 sequences (Figure 5) were deduced from the sequencing of ESTs listed in Table 3. Human FAPP1 encodes a protein of 300 amino acids, and a stop codon immediately 5′ to the predicted initiating ATG codon indicates that both the human and mouse FAPP1 protein sequences are full length. Interrogation of the human

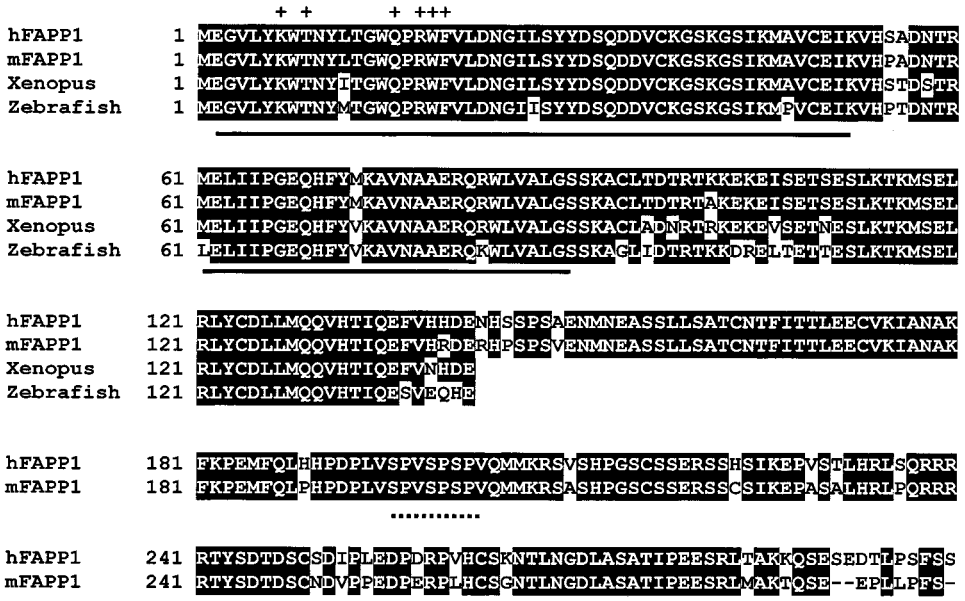


Figure 5 Amino acid sequence of human and mouse FAPP1

The alignment of the full-length human (h) and mouse (m) FAPP1 and partial *Xenopus* and zebrafish sequences are shown. The identities are shown in reversed-out white lettering on a black background. The DNA sequences of human (accession no. AF286162) and mouse FAPP1 (accession no. AF286163) are available from the NCBI database. The partial *Xenopus* and zebrafish FAPP1 sequences are predicted from the EST sequences with NCBI accession nos. AW644282 and AW174299 respectively. The amino acid residues corresponding to the PH domain are underlined with a continuous black bar, and the residues that comprise the putative SH3-domain-binding motif are indicated by a dotted line. The residues of the PH domain of FAPP1 that make up the PPBM are shown by '+'.

genome NCBI database indicated that the FAPP1 gene was located on an unmapped region of chromosome 2 (accession no. NT_003398). Analysis of the FAPP1 sequence revealed the presence of an N-terminal PH domain and a proline-rich region located towards the C-terminus that could mediate binding to SH3 domains (Figure 5). FAPP1 is likely to be expressed widely, because 27 EST clones encoding this protein were derived from several tissues (Table 3). However, FAPP1 may not be an abundant transcript, since we were unable to detect significant levels of FAPP1 mRNA expression in any tissue or cell line examined (results not shown).

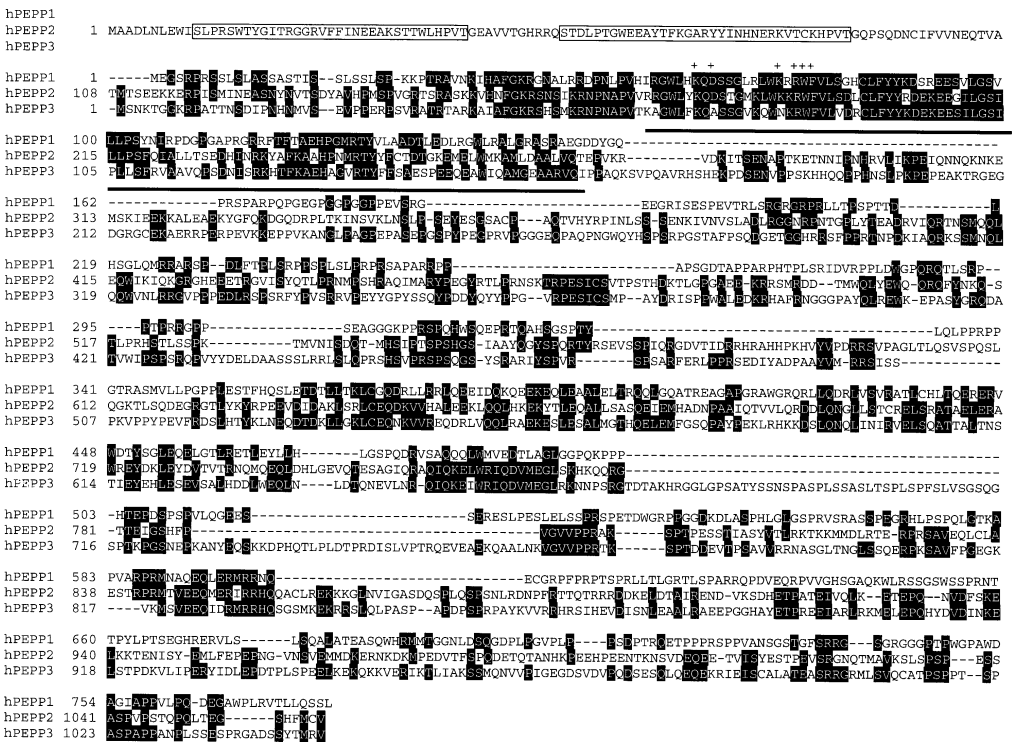
Plant AtPH1 and mammalian PEPP1 bind PtdIns3*P* specifically

Two of the PH domains that were identified, termed AtPH1 and PEPP1 (Table 1), exhibited significant affinity for PtdIns3*P*, but did not bind to any other phosphoinositide (Figure 2 and Table 2). AtPH1 is a small 145-residue *Arabidopsis* protein, the physiological role of which is unknown. It consists of one PH domain with a short N-terminal extension, and is expressed in all plant tissues [28]. PEPP1 is a novel mammalian protein, the full-length sequence of which has been deduced from sequencing of several ESTs (Table 3) and screening of a cDNA library (see the Experimental section). The PH domain of PEPP1 is located at the N-terminal region of PEPP1, and there are no other obvious functional motifs. Analysis of the NCBI human genome database shows that the *PEPP1* gene is located on an unmapped region of chromosome 19 (accession no. AC026803). The tissue distribution of PEPP1 mRNA was first investigated by Northern blot analysis, which indicated that PEPP1 was either not expressed or only expressed to a very low level in the panel of 12 tissues that we examined (Figure 6B). We also performed a Northern blot analysis using a panel of eight different human cancer cell lines (Figure 6B). Interestingly, PEPP1 mRNA was expressed at very high levels in a melanoma cancer cell line as a 3 kb fragment, but was not significantly expressed in the other seven non-melanoma cancer cell lines that were investigated (Figure 6B). Further evidence to suggest that PEPP1 may be selectively expressed in melanoma or melanocytes is provided by the fact that the three human EST clones encoding PEPP1 that we have identified thus far are derived from either a melanoma or a melanocyte cDNA library (Table 3).

Interrogation of the NCBI database with the PEPP1 sequence revealed two other proteins that appear to be related isoforms of PEPP1, termed PEPP2 and PEPP3 (Figure 6A). The identity between these proteins is most notable in the PH domain, especially the region that encompasses the PPBM, as well a region of 30 amino acids that proceeds the PH domain. PEPP1, PEPP2 and PEPP3 are poorly conserved in the region C-terminal to the PH domain (Figure 6A). PEPP2, but not PEPP1 or PEPP3, also possesses two WW domains [29] in a region N-terminal to the PH domain (Figure 6A). PEPP2 might be more widely expressed than PEPP1, since Northern blot analysis shows that PEPP2 mRNA is present in high levels in heart and kidney, and is also expressed at a lower level in other tissues. PEPP2 is also expressed in many of the cancer cell lines that were examined (Figure 6B). PEPP3 might not be an abundant transcript, since we were unable to detect significant levels of PEPP3 mRNA expression in any tissue or cell line examined (results not shown). The four PEPP3 ESTs that are present in the database are derived from brain, colon, mammary gland and skeletal muscle (see the Experimental section).

Centaurin-β2 is a PtdIns(3,5)P_2-binding protein

Human centaurin-β2 is an uncharacterized 778-amino-acid protein (cloned by T. Jackson and colleagues, University College



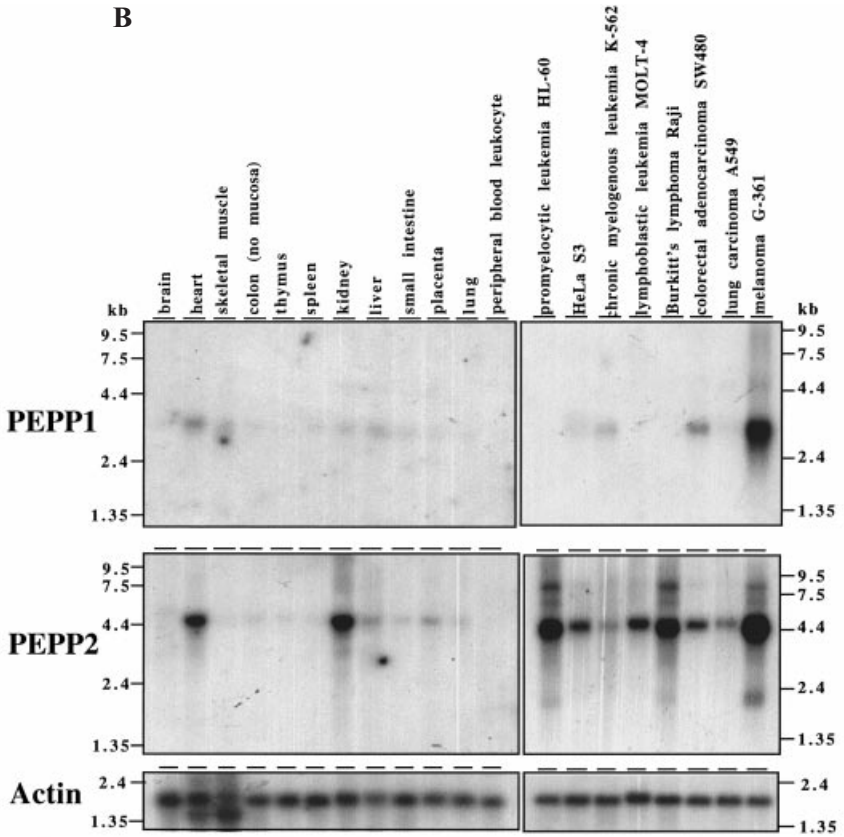


Figure 6 For legend, see opposite page

London; accession no. CAB41450), possessing a PH domain (residues 267–363) followed by a putative ARF GAP domain (residues 399–520) and three ankyrin repeats at its C-terminus. The PH domains of both mouse and human centaurin-β2 possess an asparagine residue instead of a lysine or arginine at the third conserved position of the PPBM (Table 1). The PH domain of mouse centaurin-β2 exhibited a moderate affinity for PtdIns(3,5)P_2, but did not bind to any other phosphoinositide tested (Figure 2). Centaurin-β2 is likely to be a widely expressed protein, because 12 EST clones encoding it were derived from several tissues, and Northern blot analysis indicated that mouse centaurin-β2 was expressed as a 4.5 kb fragment in all tissues investigated (results not shown).

DISCUSSION

The PH domains identified thus far that bind specifically to PtdIns(3,4,5)P_3, or to PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, possess a PPBM (Table 1). However, the finding in the present study that PH domains possessing a perfect or near-perfect PPBM consensus do not always interact with PtdIns(3,4,5)P_3 specifically emphasizes that residues lying outside the PPBM also influence the interaction of many PH domains with phosphoinositides. Structural studies also show that residues lying outside of the PPBM also form direct contacts with the inositol phosphate moieties of phosphoinositides [2,13,20]. Previous studies have demonstrated that phospholipase Cδ_1, which also possesses a PPBM, does not bind to PtdIns(3,4,5)P_3 with a high affinity [20]. It has been proposed that, in this case, the short loop between the β1 and β2 strands of the PH domain of phospholipase Cδ_1 compared with that found in other PH domains that bind to PtdIns(3,4,5)P_3 may account for this observation [20]. It therefore seems unlikely that it will be possible to predict the lipid-binding specificity of a PH domain on the basis of its amino acid sequence alone.

There has been considerable debate as to whether PtdIns(3,4)P_2 regulates the same physiological processes as PtdIns(3,4,5)P_3, because it is formed as a breakdown product of PtdIns(3,4,5)P_3 and many of the PH domains that interact with PtdIns(3,4,5)P_3 also bind to PtdIns(3,4)P_2 (see the Introduction). However, the finding that agonists, such as hydrogen peroxide [30], and cross-linking of platelet-integrin receptors [31] elevate PtdIns(3,4)P_2, without increasing PtdIns(3,4,5)P_3, suggest that PtdIns(3,4)P_2 may be able to regulate physiological processes distinct from those controlled by PtdIns(3,4,5)P_3. TAPP1 and TAPP2 (Figure 3) are the first proteins to be identified that interact with PtdIns(3,4)P_2 specifically, and may therefore be key mediators of cellular responses that are regulated specifically by this second messenger. Although there are no apparent homologues of TAPP1 and TAPP2 present in the completely sequenced genomes of *Drosophila*, *Caenorhabditis elegans* or *Saccharomyces cerevisiae*, there are ESTs encoding TAPP1 derived from zebrafish and chickens (Table 3).

Further studies are required to characterize the physiological role(s) of TAPP1 and TAPP2. It should, however, be noted that the lipid-binding properties of TAPP1 and TAPP2 *in vitro*, as well as the other PH-domain-containing proteins that we have characterized in the present study, could differ from their binding specificities *in vivo*. Furthermore, the possibility cannot be eliminated that the inositol polyphosphate head groups of the phosphoinositides, rather than the phosphoinositides themselves, could be the natural ligands for these proteins. If future studies *in vivo* do confirm that PtdIns(3,4)P_2 is the natural ligand for TAPP1 and TAPP2, it is possible that these proteins could function as adaptor proteins to recruit proteins that interact with them to cellular membranes in response to extracellular signals that lead to the generation of PtdIns(3,4)P_2. The N-terminal PH domains of TAPP1 and TAPP2, rather than interacting with lipids, might mediate protein–protein interactions, since they did not interact with any phosphoinositide that we tested (Figure 4A). TAPP1 and TAPP2 could also potentially interact with proteins containing PDZ domains via their C-terminal Ser-Xaa-Val residues, and TAPP2 might bind to SH3 domains via two proline-rich motifs located towards its C-terminus.

To our knowledge, the only PH domain previously shown to bind PtdIns4*P* with some specificity is derived from a plant PtdIns 4-kinase, which also interacts weakly with PtdIns(4,5)P_2 [32]. In contrast, FAPP1 (Figure 5) only binds PtdIns4*P*, and does not interact with PtdIns(4,5)P_2 (Figure 2 and Table 2). A key role for PtdIns4*P* in mammalian cells is to act as an intermediate in the synthesis of PtdIns(4,5)P_2. Apart from a PH domain and a putative SH3-binding proline-rich motif, FAPP1 does not possess a catalytic domain that would indicate a role in regulating the synthesis or breakdown of PtdIns4*P* in cells. There are no apparent homologues of FAPP1 in *Drosophila*, *C. elegans* or *S. cerevisiae*; however, ESTs encoding FAPP1 have been identified in zebrafish and *Xenopus* (Figure 5 and Table 3).

Genetic studies performed in yeast have demonstrated that PtdIns3*P* plays an important role in regulating Golgi-to-vacuole or Golgi-to-lysosome membrane trafficking, as well as endosome function [33]. Several proteins (e.g. early-endosomal antigen-1) regulating these processes have been found to interact with PtdIns3*P* via a particular type of zinc-finger domain (known as the FYVE domain) [34]. To our knowledge, the only other PH-domain-containing protein other than PEPP1 and AtPH1 previously reported to interact with PtdIns3*P* is phospholipase Cβ1 [35]. However, phospholipase Cβ1 might be less specific for PtdIns3*P* than PEPP1 and AtPH1, because it also possessed significant affinity for PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 [35]. The evidence indicates that phospholipase Cβ1 might be recruited to plasma membranes via an interaction of its PH domain with both PtdIns3*P* (or other phosphoinositide) and the $G_{\beta\gamma}$ regulatory subunits [35,36].

A potentially interesting feature of PEPP1 is that its expression might be restricted to melanoma and or melanocytes, as Northern blot analysis indicated that PEPP1 was expressed at very high levels in a melanoma cell line, but not in seven other non-melanoma cancer cell lines, or 12 tissues, that were investigated (Figure 6B). Further work is required to determine whether PEPP1 expression is elevated in all melanoma cells compared with normal melanocytes. It is interesting that a closely related

Figure 6 Amino acid sequence and tissue distribution of PEPP1

(**A**) The alignment of the full-length human sequences of PEPP1, PEPP2 and PEPP3 are shown. The identities are shown by reversed-out white lettering on a black background. The DNA sequences of human (h) PEPP1 (accession no. AY007233) and hPEPP3 (accession no. NM_014935) are available from the NCBI database. The amino acid residues corresponding to the PH domain are indicated underlined with a continuous line, and the region of homology preceding the PH domain is underlined with a broken line. The residues of the PH domain of PEPP1 that make up the PPBM are highlighted with ' + ' signs, and the WW domains of PEPP2 are enclosed in boxes. (**B**) The partial cDNA for PEPP1 and PEPP2 shown above was labelled with [^{32}P]α-dATP, using random primers, and used to probe a Northern blot containing polyadenylated RNA isolated from the indicated human tissues and cancer cell lines. The blot was washed and autoradiographed. The PEPP1 probe was observed to hybridize with a 3 kb message in the melanoma G-361 cell line, and the PEPP2 probe hybridized with a 4.6 kb message.

homologue of PEPP1, termed PEPP2, appears to be more widely expressed (Figure 6B). PEPP2 and PEPP3 possess a very similar sequence surrounding the PPBM of their PH domains, indicating that they may also interact with PtdIns3*P*.

Plant cells contain high levels of PtdIns3*P*, as well as PtdIns(3,4)P_2, but no PtdIns(3,4,5)P_3 has been detected [37], consistent with the apparent lack of Class 1A PI 3-kinases in plants. AtPH1 is the first plant protein that has been shown to interact specifically with PtdIns3*P*, and may play an important role as an adaptor protein in regulating signalling processes in plants that are mediated by PtdIns3*P*. The only other plant PH domain containing protein previously shown to interact with PtdIns3*P* is the plant homologue of PDK1 termed AtPDK1; however, this also interacts with a similar affinity with other phosphoinositides [18]. There are no apparent homologues of PEPP1 or AtPH1 in *Drosophila*, *C. elegans* or *S. cerevisiae*.

The ARF family of GTP-binding proteins regulate membrane trafficking and the actin cytoskeleton [38]. A family of ARF GAP proteins, collectively termed centaurins, have been identified, and all possess one or more PH domains and an ARF GAP catalytic domain [39]. The PH domain on centaurin-α1 interacts with PtdIns(3,4,5)P_3, and centaurin-α1 is recruited to cell membranes after PI 3-kinase is activated [8]. Recently, centaurin-β4 has been shown to be activated by the interaction of its PH domain with PtdIns(4,5)P_2 and, in contrast with centaurin-α1, does not bind to PtdIns(3,4,5)P_3 [40]. The finding in the present study that the uncharacterized ARF GAP protein named centaurin-β2 interacts with PtdIns(3,5)P_2, albeit with only a moderate affinity, suggests that centaurin-β2 may be regulated by this lipid. Further investigation is required to establish whether PtdIns(3,5)P_2 can lead to the activation of centaurin-β2. No protein has been shown previously to interact specifically with PtdIns(3,5)P_2, and the physiological processes regulated by this lipid are not known. In yeast, PtdIns(3,5)P_2 is generated in response to osmotic stress [41] by phosphorylation of PtdIns3*P* at the D-5 position by a kinase termed Fab1 [42,43].

In summary, this paper describes a group of novel PH-domain-containing proteins that possess interesting phosphoinositide-binding specificities. TAPP1, TAPP2, FAPP1 PEPP1, PEPP2, PEPP3 and AtPH1 might function as adaptor molecules, since they possess no obvious catalytic moieties. In order to define the physiological processes that are regulated by the PH-domain-containing proteins described in the present paper, it will be important not only to knock out these proteins in cells and mice, but also to identify the proteins that they interact with.

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